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Clinical and Experimental

Magnesium Deficiency After Prolonged Parenteral Fluid Administration and After Chronic Alcoholism Complicated by Delirium Tremens. Edmund B. Flink, M.D., Ph.D., Francis L. Stutzman, M.D., Ph.D., Arthur R. Anderson, M.D., Theodore Konig, M.D., and Robert Fraser, M.D., Minneapolis, Minn. ---------- 169

Experimental Studies of Circulatory Derangement Following the Massive Transfusion of Citrated Blood. Nobuyuki Nakasone, M.D., Elton Watkins, Jr., M.D., Charles A. Janeway, M.D., and Robert E. Gross, M.D., Boston, Mass. ---------- 184

Platelet Transfusions Utilizing Plastic Equipment. Frank H. Gardner, M.D., Doris Howell, M.D., and Erwin O. Hirsch, M.D., Boston, Mass. ---------- 196

Studies on the Normal Serum Panagglutinin Active Against Trypsinated Human Erythrocytes. III. Panagglutinin Titers in Infants and Young Children. Theodore H. Spaet, M.D., and Marcia Anderson, M.D., San Francisco, Calif. ---------- 208

Measurement of Erythrocytic Diameters With Phase Microscopy. Edna H. Tompkins, M.D., Boston, Mass. ---------- 212

Artifactual Differences in the Distribution of T-1824 and I^{131}-Labeled Albumin Resulting From Mixing Prior to Administration. Captain Norbert Freinkel, Medical Corps, United States Army Reserve, Captain George E. Schreiner, Medical Corps, United States Army Reserve, Captain John W. Athens, Medical Corps, United States Army Reserve, C. W. Hiatt, Ph.D., and Sydney Breese, M.S., Washington, D. C. ---------- 215

The Cytology of the Degenerating Leukocyte. John F. Jackson, M.D., Philadelphia, Pa. 227

Characteristic Cellular Changes in Epithelial Cells in Pernicious Anemia. Ruth M. Graham, B.S., and Maud H. Rheault, Boston, Mass. ---------- 235

Optical Isomers of Cysteine in the Prevention of Leukopenia Induced by Nitrogen Mustard. Austin S. Weisberger, M.D., and John P. Storaasli, M.D., Cleveland, Ohio 246

Serologic Tests for Syphilis in Infectious Mononucleosis. Chris J. D. Zarafonetis, M.D., Philadelphia, Pa., and John F. Kent, Ph.D., Washington, D. C. ---------- 253

Precipitins in Human Histoplasmosis. S. B. Salvin, Ph.D., and M. L. Furcolow, M.D., Hamilton, Mont. ---------- 259

(Contents continued on inside front cover)

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Contents—*(Continued from front cover)*

Concentration of Antibiotics in the Brain. William E. Wellman, M.D., Henry W. Dodge, Jr., M.D., Fordyce R. Heilman, M.D., and Magnus C. Petersen, M.D., Rochester, Minn. With the technical assistance of Dorothy Rose ---------- 275

A Simplified Method for Testing Sulfonamide Resistance of Group A Streptococci. Armine T. Wilson, M.D., Wilmington, Del. ---------- 280

Studies on the Propagation In Vitro of Poliomyelitis Viruses. V. The Application of Strain HeLa Human Epithelial Cells for Isolation and Typing. Jerome T. Syverton, M.D., William F. Scherer, M.D., and Paul M. Elwood, M.D., Minneapolis, Minn. ---------- 286

The Hemolytic Effect of Primaquine. I. The Localization of the Drug-Induced Hemolytic Defect in Primaquine-Sensitive Individuals. Raymond J. Dern, M.D., Ph.D., Irwin M. Weinstein, M.D., George V. LeRoy, M.D., David W. Talmage, M.D., and Alf S. Alving, M.D., Chicago, Ill. ---------- 303

Laboratory Methods

An Instrument and Method for Rapid, Dependable Determination of Freezing-Point Depression. Robert L. Bowman, M.D., Hillary V. Trantham, and Patricia A. Caulfield, B.A., Bethesda, Md. ---------- 310

A Rapid Method for the Determination of B_{12} Binding Power in the Gastric Juice. Richard S. Yamamoto, A.M., and Bacon F. Chow, Ph.D., Baltimore, Md. ---------- 316

The Demonstration of Fat Emboli in Tissue Sections Using Phosphin 3R, a Water-Soluble Fluorochrome. Leonard F. Peltier, M.D., Ph.D., Minneapolis, Minn. ---------- 321

A Continuous Intravenous Infusion Apparatus for the Unanesthetized Dog. Philip Whittlesey, M.D., Baltimore, Md. ---------- 324

A Practical Accurate Method for Measuring and Recording the Activity of Proteolytic Enzymes. Jack G. Bishop, M.S., and Alfred W. Richardson, Ph.D., Bloomington, Ind. ---------- 327

A Simplified Technique for Blood Volume Determinations Using I^{131}HSA. Theodore Fields, M.S., Ervin Kaplan, M.D., and Maybelle Terril, R.N., Hines, Ill. ---------- 332

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CLINICAL AND EXPERIMENTAL

MAGNESIUM DEFICIENCY AFTER PROLONGED PARENTERAL FLUID ADMINISTRATION AND AFTER CHRONIC ALCOHOLISM COMPLICATED BY DELIRIUM TREMENS*

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THE manifestations of magnesium deficiency have been well defined in various species of animals either occurring spontaneously or experimentally,[1-11] but there has not been a clear definition of the manifestations of magnesium deficiency in man. There have been many reports of low serum magnesium concentrations without any characteristic symptoms.[12-20] Tremor associated with low serum magnesium concentrations and with relief from use of magnesium salts given orally has been reported.[21, 22] Delirium tremens has been treated empirically with magnesium sulfate given parenterally with success.[23, 24] In the present paper we shall report upon a group of alcoholic patients with delirium tremens and nonalcoholic patients with tremor related to magnesium depletion. The symptoms of most of these patients were relieved by administering magnesium salts intramuscularly.

MATERIALS AND METHODS

Magnesium determinations were performed by the method of Simonsen, Westover, and Wertman.[25] Normal values by this method were established in our laboratory. The mean of

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120 determinations of serum magnesium on normals is 1.91 ± 0.20 meq./L.[26] and the mean concentration of magnesium in the cerebrospinal fluid is 2.40 ± 0.14 meq./L.[27]

The patients were selected from the medical, surgical, and psychiatric services of the Minneapolis Veterans Administration Hospital and the University of Minnesota Hospital. Serum calcium, phosphorus, and other indicated chemical determinations were also obtained. No instance of hypocalcemia was found but hypopotassemia and hypophosphatemia were occasionally noted. When the patients were able to write, samples of handwriting were obtained serially as a record of tremor.[28] Sterile 50 per cent magnesium sulfate solution was given intramuscularly (i.m.) to most of the patients. (One gram of $MgSO_4.7H_2O$ contains 99 mg. of magnesium.) Some patients received only routine hospital and nursing care; others received aqueous adrenal cortical extract, paraldehyde, glucose, vitamins, and antibiotics.

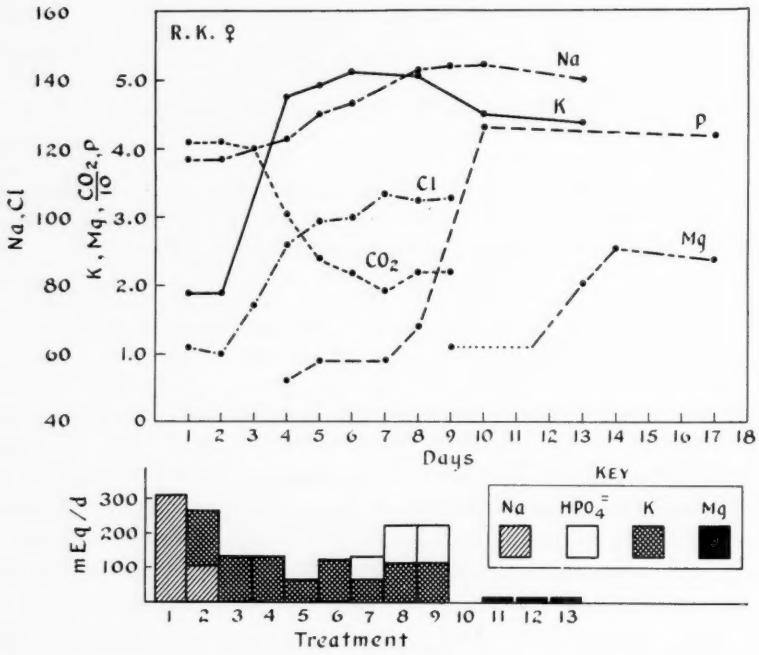


Fig. 1.—The serum concentrations are recorded by line graphs. Therapy with electrolytes is indicated below. Amounts of chloride equivalent to the sum of sodium and potassium ions were given on days 1 to 6 but are not indicated in the bar graph.

Sodium (Na), chloride (Cl), potassium (K), magnesium (Mg) and carbon dioxide (CO_2) are expressed as meq./L. and phosphorus (P) as mg. per 100 ml.

RESULTS

The interest in the relationship of magnesium deficiency to tremor began during observation of a patient briefly reported elsewhere.[29]

She was 33 years old. Three months prior to admission she was delivered of her eighth child. On the seventh post-partum day a hiatus hernia was repaired because of persistent vomiting. Following this she continued to vomit and was given parenteral fluids intermittently. She became semicomatose five days prior to admission to the University Hospital. She was cachectic, dehydrated, semicomatose, and appeared moribund on admission. Her tendon reflexes were absent, a Babinski sign was present on the left, and there was marked weakness of all muscles. She was found to have hypopotassemic, hypochloremic alkalosis, as well as

hypophosphatemia. Her serum calcium was normal. She received no sodium salts after the second day (Fig. 1). In spite of this her serum sodium concentration gradually returned to normal as her cells were repleted with potassium. As the alkalosis and hypophosphatemia were improving, she developed fibrillary twitchings, gross muscle tremor, and choreiform movements of the extremities, jaw, tongue, and facial muscles. On the sixth day she had a generalized convulsion. She was unable to talk or eat and appeared to be delirious and disoriented although it was difficult to determine her mental status because she was unable to speak clearly. At this time her serum magnesium concentration was 1.19 meq./L. Magnesium sulfate was administered intramuscularly and the muscular tremor diminished promptly; she was able to speak clearly and to eat in twenty-four hours, and the tremor soon disappeared. Electrocardiograms are the only available objective record of development and disappearance of the tremor (Fig. 2). Three days after the cessation of the tremor severe hyperesthesias and burning sensations began in her lower extremities and became progressively worse so she could not tolerate even the contact of bedclothes on her legs. Ten days later she received 40 mg. calcuim pantothenate orally and the paresthesias and hyperesthesias disappeared completely overnight.

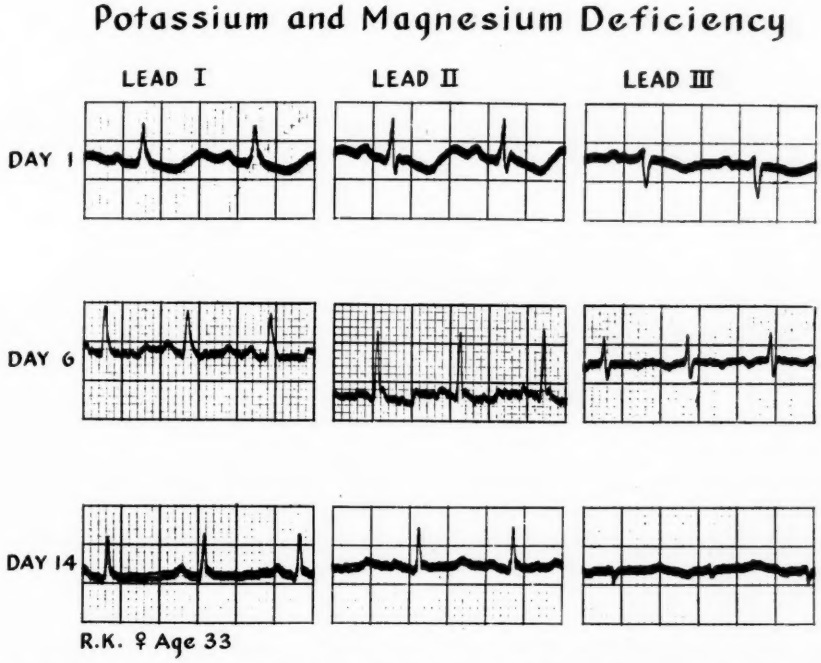


Fig. 2.—Electrocardiogram obtained on day 1 shows striking changes characteristic of hypopotassemia but no tremor. On day 6, interference by a somatic tremor is clearly evident. On day 14, or three days after starting magnesium sulfate therapy, the somatic tremor is gone. See Fig. 1 for electrolyte concentrations and therapy.

Two other nonalcoholic patients who received parenteral fluids for a prolonged period had magnesium levels of 1.35 and 1.37 meq./L. Each patient was mildly confused and had gross tremor. Magnesium therapy was apparently beneficial, but their courses were so complicated it is difficult to be certain of this.

The majority of the patients to be described are those with chronic alcoholism and delirium tremens or chronic alcoholic delirium. Tables I to III record the serum magnesium concentrations of these patients.

The cases will be divided into three groups: I. Chronic alcoholism with tremor but without frank delirium (impending delirium tremens). II. Chronic alcoholism with tremor and mild delirium. III. Chronic alcoholism with severe delirium tremens. All patients were known to have drunk excessive amounts of alcoholic beverages, and all of them had eaten poorly for several weeks to many months or even years before hospitalization. The alcoholic intake will not be discussed in the individual histories.

TABLE I. PATIENTS WITH MILD SYMPTOMS (GROUP I)

NAME	AGE	MG. CONC. MEQ./L.	$MgSO_4$ TOTAL DOSE (GRAMS)	THERAPY DURATION (DAYS)	TIME IN HOURS ELAPSED UNTIL SIGNIFICANT IMPROVEMENT OCCURRED				
					2–12	24	48	72	96
J. D.	45	1.46	36	6			x		
		1.06	64	8		x			
J. M.	37	1.14	4	1	x				
D. K.	38	1.33	24	4	x				
E. V.	48	1.39	4	2		x			
E. P.	33	1.45	12	3	x				
F. P.	44	1.64	10	1	x				
E. K.	54	1.66	4	1	x				
D. M.	38	1.67	8	1	x				
R. G.	27	1.69	9	1	x				
G. R.	62	1.35	0	0			x		
D. V.*	41	1.46	0	0			x		
H. K.	27	1.54	0	0			x		
T. G.	36	1.75	0	0		x			
	Mean	1.47							
	Standard Deviation	.20							

*Very gradual improvement. Neuropathy persisted for one month.

TABLE II. PATIENTS WITH MODERATELY SEVERE SYMPTOMS (GROUP II)

NAME	AGE	MG. CONC. MEQ./L.	$MgSO_4$ TOTAL DOSE (GRAMS)	THERAPY DURATION (DAYS)	TIME IN HOURS UNTIL SIGNIFICANT IMPROVEMENT OCCURRED				
					2–12	24	48	72	96
R. F.	42	1.45	15	1	x				
		1.44	12	3	x				
E. Ki.	49	1.53	2	1	x				
M. K.	39	1.57*	16	2	x				
S. V.†	65	1.65	8	2		x			
H. P.	39	1.29	0	0		x			
L. A.‡	41	1.32	0	0					x
D. E.	32	1.44	0	0				x	
Mean		1.46							
Standard Deviation		.12							

*Sera collected before and after therapy were inadvertently poured together; therefore, the level was actually lower than this.

†Relapse developed four days after therapy was stopped. Gradual spontaneous improvement occurred after this relapse.

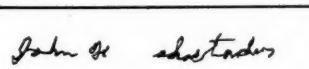
‡Convulsions occurred at twenty-four and seventy-two hours. Course otherwise not as severe as that of patients in group III.

The results of treatment of group I are recorded in Table I. The response was quicker in the patients receiving magnesium sulfate than in those receiving other forms of treatment including large doses of vitamins, adrenal cortical extract, and paraldehyde or barbiturates. It is recognized that this difference

is slight and not firmly established. Patient J. D. is described in more detail because of the observations during ammonium chloride therapy and the chronic tremor he exhibited.

J. D., aged 45 years, admitted chronic alcholism for about twenty-five years. For ten years he had a tremor which was worse when he drank heavily. He entered the hospital on two occasions and both times manifested emotional lability, irritability, restlessness and insomnia. During the first admission he was given 2 Gm. of magnesium sulfate intramuscularly four times daily with moderate improvement in tremor and marked improvement in mental state. The initial serum magnesium concentration was 1.46 meq./L.

Delirium Tremens

TIME	HANDWRITING
Before NH_4Cl 6.0 gm. daily	
24 hrs. later	
48 hrs. later NH_4Cl stopped $Mg.SO_4$ therapy started	
24 hrs. later	
4 days later	

J. D. ♂ Age 45

Fig. 3.—Signatures obtained at various times as indicated. Part of the name was obliterated to prevent identification of the patient.

On the second admission three months later, the intial serum magnesium level was 1.63 meq./L. and fourteen hours later it was 1.58. The urine magnesium concentration was 0.99 meq./L. After 6 Gm. of ammonium chloride were given per day for two days, the concentration of magnesium was 1.06 meq./L. Both observation and handwriting revealed accentuation of the tremor (Fig. 3). He became more restless and could not sleep. Two grams of magnesium sulfate were given four times every hour on the third hospital day. Two grams of magnesium sulfate were given four times daily for the next eight days. There was marked improvement in general well-being and in the tremor, but he continued to have mild tremor. His final serum magnesium concentration was 1.61 meq./L.

The results of treatment of group II are recorded in Table II. These patients treated with magnesium sulfate appear to have fared better than those who did not receive it. One patient who received no magnesium had two convulsions in the first seventy-two hours. It is noteworthy that R. F. relapsed in one week after cessation of initial therapy and responded promptly again to

another course of treatment. His course is reported in some detail below because of associated severe peripheral neuritis. S. V. also relapsed three days after stopping magnesium therapy. The relapses could probably have been prevented by more prolonged treatment.

R. F., aged 42 years, a known alcoholic and cirrhotic, was admitted to the hospital because of inability to walk or use his hands. Physical examination revealed a severe peripheral neuritis of all extremities, atrophy of the muscles of the legs and arms, pneumonia of the right middle lobe, and tremor of the hands. During the first twelve hours he was treated with penicillin, intravenous fluids, and 15 Gm. magnesium sulfate given intramuscularly. The tremor of the hands promtly disappeared. The initial magnesium concentration was 1.45 meq./L. He was treated with large doses of niacin and thiamine orally and parenterally, brewers' yeast, Betalin, multivitamins, and a high-protein and high-carbohydrate diet. The neuritis progressed to the point of wrist and foot drop until about one week after admission when a slight improvement in muscle strength and well-being was noted. On the evening of the seventh day, however, he began having hallucinations. The serum magnesium concentration at this time was 1.44 meq./L. One Gm. of magnesium sulfate was given four times daily for three days with prompt relief of the hallucinations twelve hours after therapy with magnesium was started. Calcium pantothenate was begun on this day. The wrist drop and muscle tenderness improved rapidly in the course of three days. His serum magnesium concentration was difficult to maintain at normal levels. It was 1.53 meq./L. Two months later when he was asymptomatic.

TABLE III. PATIENTS WITH SEVERE SYMPTOMS (GROUP III)

NAME	AGE	MG. CONC. MEQ./L.	$MgSO_4$ TOTAL DOSE (GRAMS)	THERAPY DURATION (DAYS)	TIME IN HOURS UNTIL SIGNIFICANT IMPROVEMENT OCCURRED				
					2–12	24	48	72	96
A. P.*	56	1.47	6	2		x			
		.87	0	0					
J. F.	37	.94						x	
W. C.	58	1.00	2	1		x			
		1.00	4	2	x				
		1.34	13	6			x		
R. R.	55	1.31	0						x
		1.21	90	9					x
		1.28	12	2		x			
M. C.†	42	1.36	10	5					0
F. L.	40	1.40	24	4	x				
E. L.†	60	1.45	2	1			0		
E. F.	54	1.52	16	2	x				
F. G.	34	1.87	8	2		x			
E. Pe.	44	—	8	1	x				
	Mean	1.29							
	Standard Deviation	.27							

*Died shortly after convulsion on day when magnesium concentration was 0.87 meq./L. This was eleven days after cessation of therapy.

†Convulsions, tremor, coma, deep jaundice, bleeding from esophageal varices were present before therapy was started. M. C. died five days later and E. L. died two days later.

Table III summarizes the course of the group III patients. Because of the more complex nature of these cases, brief summaries are given of their course.

A. P., aged 56 years, had cirrhosis and ascites and was slightly confused on admission. On the second hospital day he began to scream and required restraints. He was quite tremulous. At this time his serum magnesium concentration was 1.47 meq./L. One gram of magnesium sulfate was given intramuscularly hourly four times with decrease in confusion and

tremor. He was given 2 Gm. magnesium sulfate the next day and none subsequently. Serum concentrations were determined intermittently and progressively decreased. Eleven days after the initial treatment he was again confused and tremulous. Later that day he had a generalized convulsion and became comatose. A spinal puncture revealed grossly bloody cerebrospinal fluid with a pressure of 600 mm. of water. He died the next day. His serum magnesium concentration was 0.87 meq./L. on the day of the convulsion. His course illustrates severe relapse as result of failure to treat long enough.

W. C., aged 58 years, had been admitted before for the treatment of cirrhosis and hypopotassemic alkalosis. He had lost 19 pounds and complained of nausea and pruritus just prior to admission to the hospital. Gross tremor of the extremities and twitching of the face were evident on admission. Two days later he developed hallucinations and became disoriented. Hypopotassemic alkalosis was treated with potassium chloride given orally. The initial serum magnesium concentration was 1.0 meq./L. Two grams of magnesium sulfate were given intramuscularly and he became oriented and coherent in twenty hours. He remained oriented and felt well until eight days later when he became disoriented again, was hyperactive, had hallucinations and was noisy. Again the magnesium level was 1.0 meq./L. Two grams of magnesium sulfate were given intramuscularly and he became oriented and quiet in four hours. He remained oriented and comfortable and was discharged thirty days later. He continued to eat fairly well. Eighteen days after discharge he was readmitted because of bleeding from esophageal varices and was confused, belligerent, disoriented, and noisy. His serum magnesium concentration was 1.38 meq./L. at this time. Three grams of magnesium sulfate were given on one day and 2 Gm. were given per day for the following five days. He was quiet and oriented intermittently for forty-eight hours and after that he remained well-oriented. Bleeding from esophageal varices occurred intermittently. Jaundice became severe, and four months after the first admission he died of hepatic coma. He had extremely severe hepatic cirrhosis at autopsy. Response to the administration of magnesium had occurred on three occasions and on two of these it was quite dramatic. Relapse had occurred from a short course of therapy.

F. G., aged 34 years, had pneumonia and was treated with penicillin. On the day after admission he became agitated and later became delirious, had hallucinations, delusions, was combative, used foul language, and had to be restrained. Large amounts of paraldehyde, chloral hydrate, and sodium bromide were given on the second day, and insulin and glucose on the third day. Then 1 Gm. of magnesium sulfate was given intramuscularly hourly four times. He was quiet but not completely oriented on the morning of the fourth day (twenty-four hours after receiving the first magnesium). By that afternoon (six hours later) he was oriented and rational and continued to recover uneventfully. His initial serum magnesium level was 1.87 meq./L.

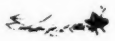
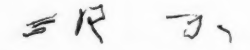
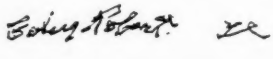
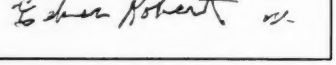
E. Pe., aged 44 years, had osteochondromatosis and was treated by operation and application of a hip spica. Parenteral fluids were given for five days because of the need for continuous gastric suction. On the sixth day he became disoriented, combative, started screaming, swearing, and using foul language. Restraints were required. Paraldehyde and adrenal cortical extract were used without apparent effect. The next day he was worse. Unfortunately an initial blood specimen was lost. Two grams of magnesium sulfate were given intramuscularly every hour four times. He was quiet in two hours and by the time of his last injection he was oriented and able to eat. He recovered uneventfully.

F. L., aged 40 years, was admitted on the orthopedic service because of pain in the left hip. He was extremely tremulous, had hallucinations and delusions, and was disoriented. Restraints were needed. The initial serum magnesium concentration was 1.4 meq./L. Two grams of magnesium sulfate were given intramuscularly every two hours four times. There was a marked decrease in tremor six hours after the first dose and he was oriented as to time and place. Magnesium sulfate (2 Gm. four times daily) was given for four days.

E. F., aged 54 years, boasted of the consumption of up to two quarts of whiskey per day. He entered on the surgical service because of an arterial occlusion affecting the left leg. A lumbar sympathectomy was performed, and on the seventh hospital day he developed delirium

tremens. He had received parenteral fluids including potassium chloride and adrenal cortical extract. He continued to get worse, developed a high fever, and was unable to speak because of gross muscle tremor. He was unable to sign his name clearly (Fig. 4). His condition was critical. The serum magnesium level was 1.52 meq./L. Two grams of magnesium sulfate were given intramuscularly hourly four times. Five hours after the first dose of magnesium he was oriented, asked for medication for pain, and handwriting was quite legible, though shaky. Magnesium sulfate was given for two days but then it was stopped. Two days after stopping magnesium he appeared to become worse, was confused, and shaky. Then 2 Gm. magnesium sulfate were given four times a day for nine days. He again improved quickly and soon became ambulatory.

Delirium Tremens and Arteriosclerosis Obliterans

TIME	HANDWRITING
Before $Mg.SO_4$ Therapy Started	
4 hrs. later	
28 hrs. later $Mg.SO_4$ therapy stopped	
24 hrs. later $Mg.SO_4$ therapy started	
9 days later	

E.F. ♂ Age 54

Fig. 4.—Signatures obtained at various times as indicated. Again, part of the name has been obliterated.

J. F., aged 37 years, had been shaky since 1943. Just before admission he had a convulsion and bit his tongue. He was jaundiced and had clinical and laboratory evidence of cirrhosis. Examination revealed that he was confused, apprehensive, combative, and had marked tremor of all muscles of the body and face. Hallucinations, delusions, screaming, and the use of foul language clearly indicated his delirium. Restraints were necessary. The initial serum magnesium concentration was 0.94 meq./L. One gram of magnesium sulfate was given intramuscularly four times on the second day and 1 Gm. twice the third day. He remained delirious for seventy-six hours. His serum phosphorus was also low (1.7 mg./100 ml.), so he was given a solution intravenously containing phosphate ions (6 meq.) seventy-six hours after the first magnesium. He had been given potassium chloride, calcium gluconate, and 20 ml. of adrenal cortical extract on the second and third days. He became quiet and oriented at noon on the fourth day just after being given the intraventous solution containing phosphate ions. It is difficult to be certain of the agent or agents responsible for his recovery, but it is probable that he had both magnesium and phosphate deficiency. His magnesium concentration was 1.98 meq./L. the day he became oriented. The level gradually fell in eleven days to 1.19 and 1.17 and he was very tense and tremulous, so he was given 1 Gm. of magnesium sulfate intra-

muscularly for five days. He continued to have a mild tremor of his hands just as patient J. D. did and his serum level also remained low, from 1.45 to 1.50 meq./L. He has pulmonary tuberculosis for which he is now receiving sanatorium care.

R. R., aged 55 years, was admitted for treatment of alcoholic cirrhosis associated with ascites and edema. He was given a low-salt diet. On the tenth day 12 Gm. of ammonium chloride orally and 2 ml. of Mercuhydrin intramuscularly were given and continued daily for four days with a loss of 7 pounds of weight. On the fourth day of this therapy he became very confused and later disoriented and developed muscle tremor. He was comatose for a short while. Ammonium chloride was discontinued. At this time his serum magnesium concentration was 1.31 meq./L. Cerebrospinal fluid obtained at this time was entirely normal but the magnesium concentration was 1.89 meq./L. of fluid. He gradually improved in the next four days without specific therapy and his magnesium concentration was 1.14 meq./L. Then he was given a total of 16 Gm. ammonium chloride in two days, and the tremor and mental confusion recurred. His magnesium concentration was 1.21 meq./L. The following nine days he was given an average of 2 Gm. magnesium sulfate five times daily intramuscularly. He recovered slowly and his mental status improved again. Twenty-eight days after the last ammonium chloride administration another trial was begun. This time he received only 6 Gm. and the whole train of symptoms recurred. He was given 1 Gm. magnesium sulfate four times a day simultaneously, but the tremor and confusion recurred anyway and he remained confused and lethargic for four days. At the onset of confusion his serum magnesium concentration was 1.59 meq./L. Ammonium chloride was stopped. He was better for two days but magnesium sulfate was stopped and he spontaneously lapsed into the former state. Then the magnesium concentration was 1.28 meq./L. Magnesium sulfate was given again and this time he was much better twenty-four hours later. His mental condition has remained somewhat confused though much better than during his episodes of delirium. It is probable that he has chronic alcoholism with organic brain damage.

DISCUSSION

Spontaneously occurring magnesium deficiency in cattle was reported by Sjollema[4] in 1932. Grass tetany (or staggers) usually occurs the first few weeks after cows are put out to graze in the spring, but a few cows develop the same symptoms after calving. The cows are restless and nervous, graze away from the herd, lack appetite, and have muscle twitching, unsteady gait, spreading of the hind limbs, gnashing of teeth, and rolling of eyes with an anxious and wild look. Some cows low incessantly. Soon after the first symptoms, tonic and clonic convulsions appear. This is followed by a paretic or comatose state. Serum magnesium concentrations usually ranged from 1.0 to 0.2 mg./100 ml. with an average of 0.5 mg./100 ml. Calcium chloride and magnesium chloride given intravenously caused rapid improvement, though animals still were nervous and without appetite for a few days. One of the characteristics of the new grass is a very high nitrogen content compared with hay.

In 1932 Kruse, Orent, and McCollum[1, 2, 3] reported the results of experimental magnesium deficiency in rats. Young weaned rats developed severe deficiency with convulsions and died in eighteen to twenty-one days. There was a marked growth disturbance. These rats had marked vasodilation in exposed skin areas. The same investigators[2] found that dogs showed less irritability but more impaired nutrition than rats.

Tufts and Greenberg[8] recognized two phases of deficiency: (1) vasodilation, hyperemia, hyperexcitability, and (2) marked malnutrition, cachexia, and

kidney damage. They found that high calcium intake increases the severity and rapidity of magnesium deficiency.

Duncan, Huffman, and Robinson[5] described tetany in calves maintained solely on milk. The calves had low magnesium concentration but normal calcium and phosphorus. The manifestations were similar to grass tetany in cows.

Colby and Frye[30, 31] studied factors which enhance magnesium deficiency. They found that large intake of either calcium or potassium, of calcium and potassium, or of protein enhances magnesium deficiency. This observation helps explain both the grass tetany in cows and the milk tetany in calves and confirms Tufts' and Greenberg's[9] observations.

Menaker and Kleiner[32] found that rats which had lost 25 per cent of their weight because of protein depletion gained back very little weight on adequate protein but magnesium-deficient diet. This is similar to the effect of potassium deficiency.

The body magnesium decreases from a control of 40 mg./100 Gm. fresh carcass in rats to 25 mg./100 Gm. in fifteen days on a magnesium-deficient diet. The concentration of brain and muscle magnesium decreases significantly in the second phase of deficiency.[8] Cotlove and co-workers[33] also found definite decrease in magnesium of muscles. In spite of adequate potassium intake, the muscle potassium was also low.

The recognition of magnesium deficiency in human beings is less sharply defined. However, as early as 1934, Hirschfelder and Haury[21] reported the occurrence of muscular twitchings and convulsions in 7 patients who had low serum magnesium concentrations. These patients had either severe renal disease or toxemia of pregnancy. Magnesium sulfate was given by mouth and twitchings stopped in 4 patients. They concluded: "There is a clinical syndrome of low magnesium (hypomagnesemia) accompanied by muscular twitching or by convulsions."

Miller[22] reported the occurrence of tremor and muscle twitching in a 6-year-old boy with magnesium deficiency. Very troublesome muscle twitching interfering with finer movements and particularly with writing was relieved by oral administration of magnesium sulfate. The lowest serum concentration was 0.5 meq./L. in this child.

Denis and Talbot[17] reported low magnesium and calcium values in tetany occurring in infancy, but they focussed all their attention on the calcium values.

Martin, Mehl, and Wertman[16] reported 16 patients with magnesium levels under 1.0 meq./L. None of these had tetany. They were impressed with the rarity of tetany and had seen it only once with hypomagnesemia, and that patient also had low serum calcium. They listed the conditions in which low serum magnesium concentrations have been found: postoperative states with use of magnesium-free intravenous fluids, malignancy, diabetic acidosis during treatment, chronic renal disease, recovery phase of acute renal insufficiency, congestive cardiac failure, epilepsy, lupus erythematosis, hyperthyroidism, and pancreatitis. That few clinical symptoms have been ascribed to hypomag-

nesemia is also emphasized by the discussion of magnesium metabolism in the most recent edition of a textbook on diseases of metabolism. "Little is known as yet of its function in the human organisms and of the relation of disturbance of magnesium metabolism to clinically observable abnormalities in man."[12]

Haury[19] found low magnesium concentration in 16 of a group of 66 patients with asthma. Haury and Cantarow[18] found low levels in some patients with diabetes, toxemias of pregnancy, neoplasms, chronic arthritis, and epilepsy.

Gardner and co-workers[20] studied 16 infants with neonatal tetany. All were fed cow's milk. In one patient the serum magnesium concentration decreased to 0.34 meq./L. at one time. The high phosphorus content of milk was thought to account for the low calcium and magnesium concentrations. The emphasis of this paper is the hypocalcemia which developed.

The results of the observations reported in this paper duplicate the findings in naturally occurring and in induced or experimental magnesium deficiency in animals. The most characteristic clinical feature is the occurrence of bizarre involuntary muscle activity manifested as gross tremor, athetoid, and choreiform movements of the extremities and twitching of the face. Convulsions occurred in some patients. As the name implies delirium also occurs. Some of the patients reported cannot be said to have been actually delirious, but all who were not delirious were extremely apprehensive and anxious. Varying degrees of confusion, disorientation, hyperactivity, hallucinations, and delusions occurred. Stupor and coma occurred in some of the patients with hepatic cirrhosis. The lowest magnesium concentrations occurred in very ill patients and there is some correlation of severity of manifestations and the magnesium concentration. Handwriting was found to be a practical way of recording the tremor and progress of the illness.

It is well known that most patients with delirium tremens recover spontaneously when they are given sedation and begin eating. There are some patients, however, who develop convulsions and become progressively weaker and die. The prognosis in delirium tremens depends in part on the associated condition, but mortality rates have varied from 5 per cent to 33 per cent as stated by Cline and Coleman.[35] Some of the patients in this group were not given magnesium but made fairly prompt recovery. There were, however, some patients (W. C., E. F., E. Pe., F. G., J. D., E. V.) who were getting progressively worse on other forms of therapy and whose recovery began when magnesium was administered.

All patients with serum magnesium concentrations below the normal range do not have the symptoms or signs described above. Even after recovery from delirium tremens some patients have subnormal levels; this was especially true in patients with associated cirrhosis of the liver. Low serum magnesium concentrations occur in animals, too, without symptoms.[34] Patients with cirrhosis may have low magnesium concentrations without demonstrable symptoms and signs.[26] The absence of symptoms or signs when there is a low serum magnesium concentration could be compared to the absence of symptoms and signs in some patients with hypopotassemia.

All the patients studied had levels below the normal mean (1.91 ± 0.20 meq./L.) and the mean of the patients (1.40 ± 0.24 meq./L.) was significantly lower than the normal mean. This difference is highly significant statistically ($t = 12.6$, $p < 0.01$). There were a few patients with magnesium levels within one standard deviation of the normal mean. This finding may be similar to that noted in experimental magnesium deficiency by Tufts and Greenberg.[8] They observed that serum magnesium decreased sharply at first, tended to approach normal as hyperexcitability was developing, but finally fell to about half of normal in advanced magnesium deficiency. Patients in group III had the lowest mean magnesium concentration. Those in group II had lower mean concentration than group I. This is consistent with findings in experimental magnesium deficiency. The difference between the means of groups I and III is not significant statistically, however ($t = 1.58$ $p = .2$).

The fact that the hyperexcitability to noxious stimuli of magnesium-deficient animals is not immediately corrected by the injection of magnesium salts[10] and that magnesium will correct the deficient state gradually, suggests that the symptoms and signs are an index of cellular and total body depletion of magnesium rather than a manifestation of low extracellular concentration. The same phenomenon has been observed in human beings. Sometimes there has been a dramatic and almost immediate cessation of symptoms and signs, but often a period of from twelve to as long as seventy-six hours elapsed before the delirium was relieved. It is suggested, therefore, that the low serum concentration in the patients described is an expression of cellular deficit of magnesium but is not very significant in itself.

Magnesium sulfate has been used successfully for the treatment of acute and chronic alcoholic psychoses in the past because of the known sedative action of magnesium.[23, 24] Rossetti[24] speculated on the possibility of magnesium deficiency. In addition Cline and Coleman[35] used 1 to 2 ounces of saturated solution of magnesium sulfate orally as a purgative. Their results were much better than any previously experienced in their hospital. They believed that their therapeutic regimen, which also included spinal drainage, was effective because of a dehydrating effect. It is known, however, that a considerable absorption of magnesium occurs when magnesium sulfate is given orally.[21]

Magnesium deficiency has produced lesions in the cerebellum of chicks[36] and in the brain, kidney, and liver of rabbits and rats,[37] the heart in rats,[7] and in the heart, blood vessels, and diaphragm of calves.[38] The first patient (J. K.) had peripheral neuritis as did several of the chronic alcoholic patients (D. V., R. F.).

The pathogenesis of magnesium deficiency in chronic alcoholism is similar to that induced by a magnesium-deficient diet. The caloric requirement of these patients is satisfied by alcohol almost exclusively for a long time and, thus, their diet is low in magnesium. The daily requirement of magnesium for adults has been estimated to be 5.5 mg./kg. body weight/day by Chu[39] and to be from 0.35 to 0.45 Gm. daily by Coons.[40] A diet consisting mostly of distilled liquors is deficient in many essential food substances, of course.

It was found necessary to give magnesium sulfate for a number of days or there would be a relapse in the patient's condition. This happened in patients A. P., W. C., S. V., J. F., and E. F. At the present time it is recommended that 2 Gm. magnesium sulfate (50 per cent solution) be given intramuscularly four times a day for three days and then 1 Gm. four times a day for two to four days longer in the more severe instances. In mild cases when the patient eats well, magnesium therapy need not be continued past the third day. It is safe to give 2 Gm. magnesium sulfate intramuscularly every two hours four or five times on the first day. No severe local reactions have been seen, but after three or four days patients begin to complain of soreness at the site of injection. Severe renal insufficiency is a contraindication to the large doses recommended previously but not for smaller doses. As a matter of fact, magnesium therapy is indicated in patients with renal insufficiency complicated by convulsions and tremor, but small doses should be employed. Oral administration of magnesium salts has not been tried. It should be effective since approximately 40 per cent of an oral dose of magnesium appears in the urine.[21]

Ammonium chloride causes a negative magnesium balance.[41] In some patients it appeared to be related to a marked decrease in serum magnesium (J. D. and R. R.), whereas in others, including patients with cirrhosis there was no noticeable adverse effect of ammonium chloride (unpublished data). Ammonium chloride and other nitrogenous compounds have been found to cause gross muscular tremor in some patients, but not in others.[42]

Magnesium is an essential intracellular ion. Many enzyme actions require the presence of magnesium ion. Magnesium has an important function in relation to adenosine triphosphate (ATP), though this relationship is not completely understood. Magnesium influences adenosinetriphosphatase activity of myosin B.[43] Under the influence of magnesium, the ATP content of the muscle of rats is increased. The effect is due in part to a decrease in the utilization of ATP and phosphocreatine fractions of the muscle.[44] Magnesium antagonizes the electrocardiographic effects of topical application of ATP and ADP on the heart, probably by inhibiting dephosphorylation of these compounds.[45] There is a suggested relationship, then, of magnesium to energy release mechanisms.

SUMMARY

Patients receiving parenteral fluids for a long time may develop magnesium deficiency and have gross muscular tremor and delirium. Magnesium therapy alleviates these manifestations.

Patients with delirium tremens or chronic alcoholic delirium have low serum magnesium concentrations. The manifestations are usually promptly alleviated by the administration of magnesium sulfate intramuscularly in amounts which are not hypnotic.

Magnesium deficiency is probably more common than generally appreciated. Magnesium tetany appears to be manifested by gross muscular tremor, athetoid, and choreiform movements and in the severer forms by convulsions. The manifestations are different from those of hypocalcemic tetany.

The whole group of patients reported previously have manifested all of the symptoms and signs noted in magnesium-deficient animals. An individual patient may have only a few of the signs and symptoms.

The severity of the symptoms is roughly correlated with the initial magnesium concentration.

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EXPERIMENTAL STUDIES OF CIRCULATORY DERANGEMENT FOLLOWING THE MASSIVE TRANSFUSION OF CITRATED BLOOD

Comparison of Blood Treated With A.C.D. Solution* and Blood Decalcified by Ion Exchange Resin

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PRIOR to 1940, several reports indicated that reduced blood calcium ion concentration, induced by the citrate used as an anticoagulant in donor blood, could be implicated in certain odd types of transfusion reaction.[1] It was soon shown by numerous experimental and clinical studies that, to produce toxic effects, far larger amounts of citrate had to be infused with greater rapidity than would prevail with the methods of intravenous transfusion therapy in clinical use at that time.[1] In 1944, Adams and co-workers documented the limits of safe transfusion of citrated blood in dogs.[2] They concluded that "a large margin of safety is present even in massive transfusions of citrated whole blood or plasma where the administration is at a maximum rate currently employed in man (1,000 c.c. per hour)." They observed that calcium gluconate was effective in alleviating citrate intoxication. There was an inverse relationship between the rate of administration of citrated blood and the total amount of citrate producing circulatory effects.

During recent studies with a rapid-flow artificial kidney, our experiments were frequently interrupted by the death of the animal within a few minutes after the artificial perfusion was started. Additional investigation showed that such effects were probably caused by sudden infusion of excess citrate contained in the A.C.D.-treated dog blood used to fill the chambers of the artificial kidney. Since the original descriptions of the innocuous effects of citrated blood, the indications for massive transfusions have increased, and special techniques of blood administration (such as exchange transfusion, intra-arterial transfusion, and mechanical aids to the circulation) have been introduced so that the upper limits of safe rate of infusion and cumulative citrate dosage (as documented by Adams and co-workers[2]) may be exceeded. Consequently it would appear desirable to re-examine the entire question of citrate toxicity in the light of present-day transfusion practice. This report

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*Anticoagulant Acid Citrate Dextrose Solution U. S. P.

presents a group of experiments designed to study the acute circulatory derangements induced in the dog by citrate infusion under conditions similar to certain present-day transfusion practices.

EXPERIMENTAL METHODS

Adult mongrel dogs ranging in weight from 15 to 21 kilograms were used. All studies were performed under Nembutal anesthesia; the agent was administered intravenously in approximate doses of 30 mg. per kilogram of body weight, gauging the level of surgical anesthesia by loss of neck and jaw rigidity and varying depression of the conjunctival reflex. All experiments were performed with the animals horizontal in the right lateral decubitus position.

Physiologic Methods.—Simultaneous electrocardiograms and arterial pressure contours were recorded with a two-channel direct writing oscillograph and a capacitance manometer.* During each observation period of an experiment, a standard one millivolt deflection was impressed on the limb Lead II electrocardiogram, and static pressure calibrations recorded in the range of the observed pressure pulse. Arterial pressures were recorded from the unobstructed carotid artery exposed low in the neck; connection was made to the pressure transducer through a three-foot lead tube, stopcock, and large-bore hypodermic needle.

Stroke volume index was calculated from the carotid pulse contour by a modification of the second Hamilton-Remington method.[3] Computations were made on enlarged camera obscura tracings of the recorded pulse. Mean pressure was calculated by summation of the systolic and diastolic time-pressure areas graphically integrated in the stroke volume calculation. Total peripheral resistance index was calculated in c.g.s. units by means of the equation:†

$$\frac{\text{Mean Arterial Pressure (mm. Hg)}}{\text{Flow (ml. per sec. per M}^2\text{)}} \quad (1332) \quad = \quad \text{TPR Index in dyne sec. cm.}^{-5} \text{ per square meter of body surface area}$$

RESULTS

Artificial Kidney Primed With A.C.D.-Treated Blood.—Our interest in this problem was stimulated initially by the circulatory changes observed immediately following start of perfusion of normal dogs using an artificial kidney machine[4] primed with A.C.D.-treated blood. Since we wished to observe rates of rapid gas exchange across the plastic membranes of this apparatus, the vessel cannulation was designed to permit high rates of blood flow through the kidney machine. A large-bore plastic cannula, introduced through the external jugular vein into the right atrium, withdrew blood from the animal for passage into the machine, and blood was returned to the animal through

*Sanborn Twin-Viso Electrocardiograph and Electromanometer, Sanborn Company, Cambridge, Mass.

†We recognize that there may be some question as to the accuracy of the pulse contour method and the derived figure for peripheral resistance. The pulse contour method in our hands has shown consistent serial trends and has given good correlation to output figures calculated by direct Fick or dye injection methods in a limited number of observations in intact Nembutalized dogs. Peripheral resistance is calculated as an index and was not converted to the absolute value since our interest was in observing relative trends during a single experiment.

a cannula introduced through the femoral vein into the inferior vena cava. The dog was heparinized with 2 mg. of heparin per kilogram of body weight prior to start of the perfusion. Studies with this mode of connection of the artificial kidney showed no derangement of stable circulatory function if heparinized blood were used to fill the kidney or on starting and stopping rapid machine flows once the animal had adjusted to the initial mixing of a citrated prime blood. These findings indicate that the changes noted on first starting the kidney perfusion with A.C.D. blood were not due to alterations of venous return induced by the technique of external pumping.

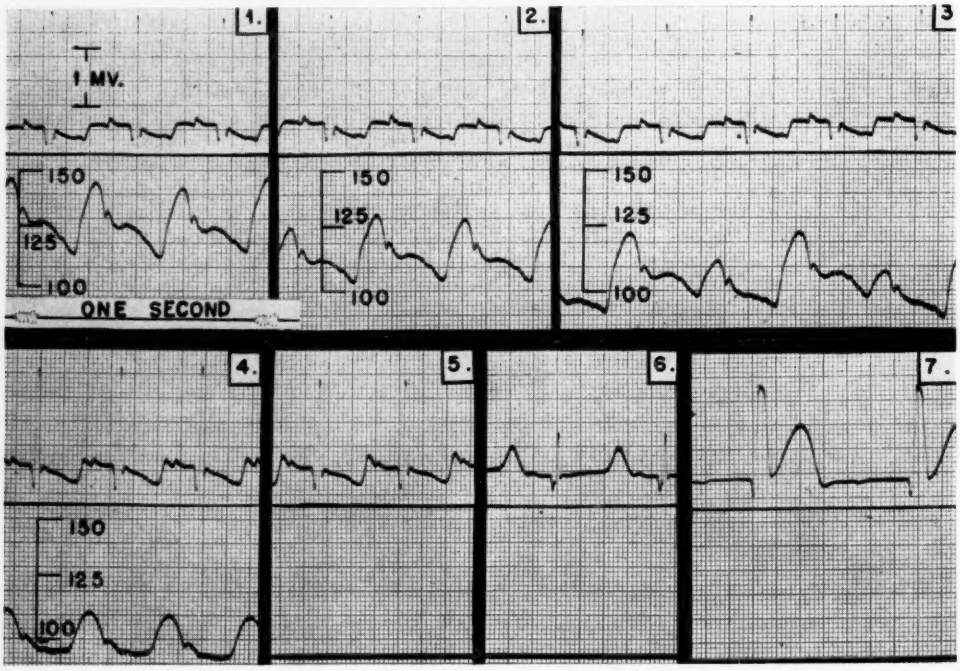

Fig. 1.—Lead II electrocardiograms and carotid arterial pressure during the start of an artificial kidney perfusion in a dog. *Segment 1*, Control tracing; *Segment 2*, 15 seconds after the start of perfusion; *Segment 3*, 30 seconds; *Segment 4*, 45 seconds; *Segment 5*, 60 seconds; *Segment 6*, 67 seconds; *Segment 7*, 11 minutes after start of perfusions and 2 minutes after intravenous injection of 9.1 mM calcium (calcium chloride 1.0 Gm.). (Pressure calibration in millimeters of mercury.)

A typical perfusion disaster is shown in Fig. 1. The animal weighed 20 kilograms, the volume of A.C.D.-treated blood priming the machine was 700 ml., and the rate of flow through the artificial kidney was 400 ml. per minute. Under these conditions, on starting the external circulation, the animal received 12.4 mM citrate in 1.8 minutes (0.357 mM/kg./min.). Over the course of one minute after the start of perfusion, there was a progressive decline of arterial pressure until no pulsations could be observed at sixty seconds (Segment 5). Earlier there was a period of pulse alternation with a heart rate of 165. Stroke volume fell and peripheral resistance rose during this period of declining arterial pressure. Electrocardiographic changes occurred while ar-

terial pressure was still sustained. These changes included: progressive prolongation of the QT interval and QT/PT ratio, progressive depression of the T wave (which was inverted in the control), and late reduction of P-wave voltage. The patterns recorded after disappearance of arterial pulsations showed a nonspecific injury pattern with high peaked T waves. The intravenous administration of calcium showed no effect when it was injected after the cessation of effective peripheral circulation.

These sequential changes in the electrocardiogram are typical of those reported previously for hypocalcemia due to various causes.[5, 6] Such a straightforward result, which might have been expected on the basis of the rapid exchange rates of citrate, indicates how our concepts of the lack of citrate toxicity were carried over into a situation in which toxicity might be expected. The trouble was easily avoided by use of other methods to maintain the priming blood in a fluid state.

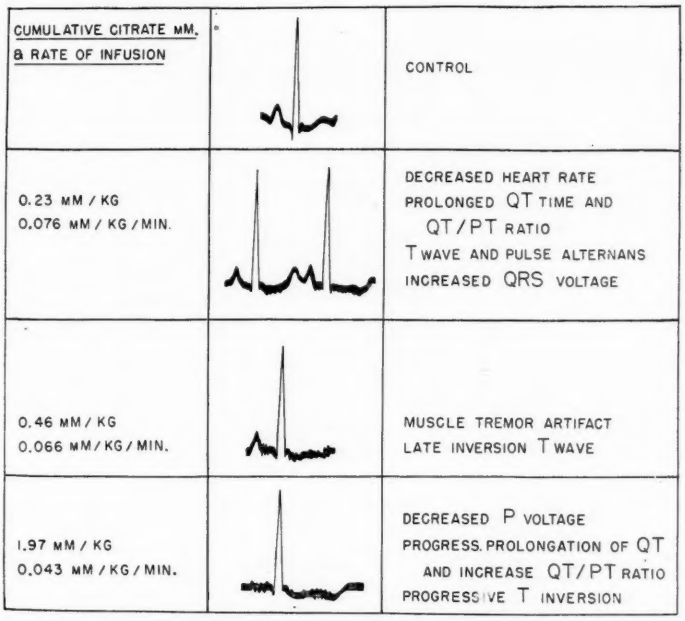
CUMULATIVE CITRATE MM.
& RATE OF INFUSION
CONTROL
0.23 MM / KG
0.076 MM / KG / MIN.
DECREASED HEART RATE
PROLONGED QT TIME AND
QT / PT RATIO
T WAVE AND PULSE ALTERNANS
INCREASED QRS VOLTAGE
0.46 MM / KG
0.066 MM / KG / MIN.
MUSCLE TREMOR ARTIFACT
LATE INVERSION T WAVE
1.97 MM / KG
0.043 MM / KG / MIN.
DECREASED P VOLTAGE
PROGRESS. PROLONGATION OF QT
AND INCREASE QT / PT RATIO
PROGRESSIVE T INVERSION

Fig. 2.—Changes in the contour of Lead II electrocardiogram during the intravenous infusion of acid-citrate dextrose solution (A.C.D. solution) in a dog.

Intravenous Infusion of A.C.D. Solution and Calcium-Citrate Solution.— To study the sequential physiologic changes in the dog during the development of citrate toxicity, we infused A.C.D. solution* intravenously and observed the animal's response. The results of a typical experiment are shown in Fig. 2. The similarity of these changes is apparent to those observed with the artificial kidney perfusion. Mechanical alternation of the pulse, with or

*A.C.D. solution having the following composition was used in these experiments: Trisodium Citrate ($Na_3C_6H_5O_7 5.5\ H_2O$)–20 Gm.; Citric Acid ($C_6H_8O_7 : H_2O$)–8 Gm.; Dextrose 23 Gm.; Water U.S.P. to make 1,000 ml. In the exchange transfusions, 250 ml. blood were treated with 35 ml. A.C.D. containing 4.4 mM of citrate; 500 ml. blood were treated with 70 ml. solution containing 8.8 mM Citrate.

without electrical alternation of the T wave, could also be demonstrated during the infusion of A.C.D. solution. When the A.C.D. solution was infused at a slower rate in other animals, comparable electrocardiographic changes could be produced, but only at higher cumulative citrate dosages. This finding is in keeping with the experimental evidence of a rapid breakdown of the citrate radical in the body.

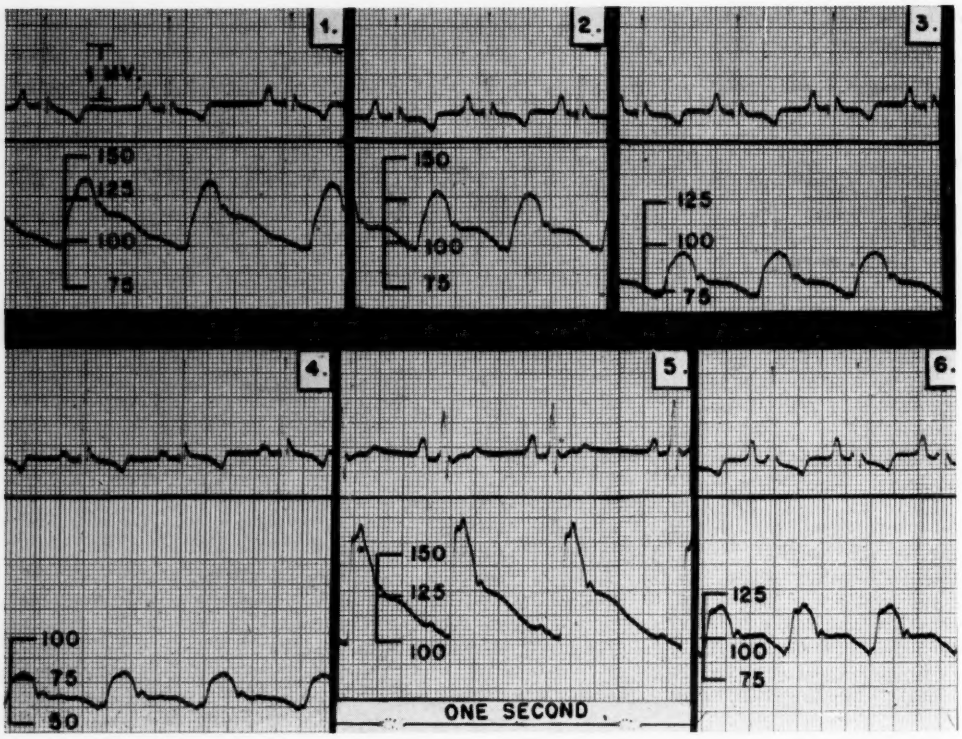

Fig. 3.—Lead II electrocardiogram and carotid arterial pressure during an exchange transfusion of A.C.D.-treated blood in a dog. *Segment 1*, Control tracings, *Segment 2*, Stable tracing following the exchange of 800 ml. blood (25 minutes, 14.4 mM total citrate, 0.96 mM per kilogram, 0.038 mM per kilogram per minute). *Segment 3*, 1,000 ml. exchange (34 minutes, 17.6 mM total citrate, 1.18 mM per kilogram, 0.035 mM per kilogram per minute). *Segment 4*, 1,250 ml. exchange (40 minutes, 22.0 mM total citrate, 1.47 mM per kilogram, 0.037 mM per kilogram per minute), exchange discontinued. *Segment 5*, 30 seconds after the intravenous administration of 1.0 Gm. of calcium chloride (9.1 mM calcium, 45 minutes). *Segment 6*, Ten minutes after calcium administration (55 minutes).

Additional evidence was obtained that these electrocardiographic changes were produced by calcium binding. Simultaneous infusion of sodium citrate and calcium chloride solutions, so that equimolar amounts of citrate and calcium were introduced simultaneously, adequately protected the animal against citrate concentrations found to be lethal when A.C.D. solution was infused alone. Electrocardiographic patterns of the type described above did not appear.

Intravenous Exchange Transfusion With A.C.D.-Treated Blood.—Several excellent reports have described the conditions during the exchange transfusion of citrated blood for the treatment of erythroblastosis. Among the changes that have been described are: transient elevations of blood citrate

concentration[7, 8]; reduction of serum calcium ion concentration[7]; and prolongation of the QT interval of the electrocardiogram.[7] It should be recognized that with the development of modern radical surgical procedures, transfusion therapy in adults may approach proportionately large volumes of citrate administration. In view of our observation that rapid rates of citrate infusion may induce circulatory changes at low cumulative citrate dosages, quantitation of the serial effects should be carried out with particular reference to the rate of infusion of citrated blood.

To study the influence of A.C.D.-treated blood in the absence of transient alterations of blood volume, exchange transfusion was accomplished by withdrawal of 250 ml. portions of blood from the dog's femoral artery into glass bottles containing 35 ml. A.C.D. solution (4.4 mM citrate) while equal volumes of A.C.D.-treated blood were being injected simultaneously into the right atrium through a polyethylene cannula. The initial 250 ml. unit of blood was withdrawn from donor animal, and successive units of the citrated blood withdrawn from the experimental animal were reinjected as the next aliquot was removed. The additional fluid volume of A.C.D. solution is not regarded as sufficient to account for the circulatory changes noted. Physiologic observations were made during stable periods when injection was not being performed.

A typical experiment is shown in Fig. 3. In this 15 kilogram animal, prolongation of the QT interval occurred early in the exchange and by the time 1,250 ml. blood had been exchanged, effects similar to the changes in the A.C.D. experiments could be noted in the electrocardiogram. There were prolongation of the Q-T interval and QT/PT ratio, depression of P voltage, and a somatic tremor artifact. Severe bronchospasm was noted at the end of 1,250 ml. exchange. At the time of development of these findings, the amount of citrate administered was 2.23 mM per kilogram at an infusion rate averaging 0.056 mM per kilogram per minute.

Arterial pressure and stroke volume showed progressive decline as the exchange progressed. Peripheral resistance was reduced to a value 44 per cent of the control.

Administration of 9.1 mM of calcium as calcium chloride produced reversion of the electrocardiographic changes and an elevation of the arterial blood pressure and stroke volume. The alteration of the pulse contour is typical of the changes elicited by calcium administration: rapid systolic ejection, development of a prominent anacrotic peak, and a wide pulse pressure. The anacrotic peak has often exceeded systolic pressure at the end of ventricular ejection. Peripheral resistance returned to control levels. Ten minutes after calcium injection, arterial pressure was sustained close to control values and the electrocardiographic pattern suggesting low calcium-ion concentration was not present. Peripheral resistance rose above control values.

Rate of exchange of citrated blood: We noted that there was an inverse ratio between the rate of intravenous infusion of A.C.D. solution and the cumulative amount of citrate required to produce equivalent electrocardiographic

changes. Consequently it was interesting to note changes in one animal early during exchange transfusion of citrated blood. This dog showed the typical changes of transient reduction of calcium-ion concentration during exchange of the first 250 ml. unit of blood at a rapid rate. Immediately after the administration of 250 ml. blood containing citrate to give a dose of only 0.22 mM per kilogram (infusion rate 0.055 mM per kilogram per minute) there was prolongation of the Q-T interval and pulse alternation. Two minutes after cessation of this small exchange, the abnormalities had disappeared. As the exchange continued, the later electrocardiographic changes appeared and persisted for longer periods after each 250 ml. exchange was completed. During 1.5 hours 2,000 ml. blood were exchanged, giving a cumulative citrate dose of 1.77 mM per kilogram at an intermittent infusion rate averaging 0.02 mM per kilogram per minute. The hypocalcemic electrocardiographic pattern was then persistent, but over the course of two hours showed gradual reversion toward normal. Pulse alternation persisted.

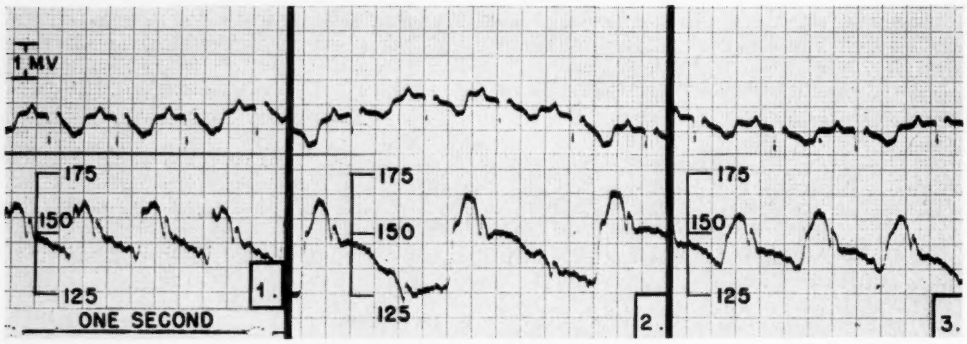

Fig. 4.—Transient changes in Lead II electrocardiogram and carotid arterial pressure in a dog early during an exchange transfusion with A.C.D.-treated blood. *Segment 1,* Control tracing. *Segment 2,* Stable tracing immediately after exchange of 250 ml. A.C.D.-treated blood (4 minutes, 4.4 mM total citrate, 0.22 per kilogram, 0.055 mM per kilogram per minute). *Segment 3,* Two minutes after *Segment 2.*

The significance of pulse alternation or of Q-T interval prolongation might be questioned at these rapid heart rates. On the other hand, these derangements have occurred in animals with relatively normal cardiovascular mechanisms, and the blood has been exchanged in such a way that reductions of circulating blood volume have not occurred. Blood would be administered clinically in these amounts for the management of severe hypovolemia or normovolemic shock, in which there is derangement of circulation through the myocardium and through organs active in the metabolism of exogenous citrate. Consequently we would question whether citrate blood is the blood replacement of choice in such emergency situations, particularly when antecedent myocardial disease, conduction abnormality, or liver disease exists.

Intravenous Exchange Transfusion With Blood Rendered Noncoagulable by Passage Across a Cation Exchange Resin. Experiments With Isolated Cation Deficiencies.—Ordinary bank blood contains an excess of citrate which will bind divalent plasma cations other than calcium. This fact suggests two

interesting possibilities. First, blood rendered fluid by citrate may be more likely to induce circulatory derangements than blood made noncoagulable by a method which removes plasma cations active in the clotting mechanism but does not introduce an excess of cation-binding agent into the body. Second, derangement of magnesium ionization in the blood stream, or differential variations in the rates of restoration of calcium and magnesium from body reservoirs, may play a role in citrate intoxication.

The introduction by Walter of a new technique of collecting blood in polyvinyl plastic bags[9] affords a method for studying these problems. Blood collected in this way is rendered noncoagulable by passage across a resin column of sulfonated polystyrene-divinyl benzene copolymer, which replaces with sodium the plasma calcium, potassium, and magnesium of the shed blood.[9] By reconstituting certain of these ions after passage of the blood across the resin column, one can study circulatory effects during exchange transfusion using blood with specific cation deficiencies.

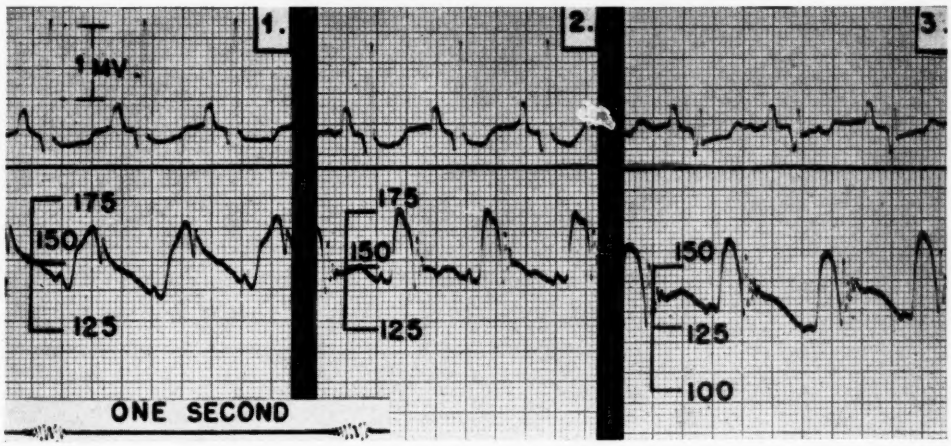

Fig. 5.—Lead II electrocardiogram and carotid arterial pressure during an exchange transfusion with blood decalcified by passage across a cation exchange resin. *Segment 1*, Control tracing. *Segment 2*, Stable tracing following completion of exchange of 1,000 ml. of blood (160 minutes). *Segment 3*, Stable tracing following exchange to 4,000 ml. blood (285 minutes). The variations in pulse contour between *1* and *2* are probably caused by change to a different type of recording cannula.

The method of exchange transfusion was similar to the method described for citrated blood. Five hundred gram portions of blood were withdrawn from the femoral artery as equal amounts were being infused into the right atrium. Multiple disposable plastic units were used to effect the exchange and passage across cation exchange resin columns.* Blood was passed across each 50 gram resin column at a rate not faster than 50 grams of blood per minute, to a total of 500 grams per column. Under these conditions 99 per cent of the calcium, 97 per cent of the magnesium, and 87 per cent of the potassium are removed from the blood.[10] After each 500 gram quantity was withdrawn, potassium or potassium and magnesium as the chloride were intro-

*B-2 Blood Pack. Fenwal Division, Macalaster Bicknell Parenteral Corporation, 234 Broadway, Cambridge, Mass.

duced into the plastic bag in concentrations of 2.5 meq. per liter of blood to obtain approximate restitution of normal plasma concentrations of these cations.

At this time a limited number of experiments have been performed with magnesium-potassium reconstitution and with potassium reconstitution. Consequently we can draw no conclusions about the influence of specific cation deficiency other than to note that far more resin-treated blood can be exchanged without deleterious circulatory effects than can be exchanged when citrate is used as the anticoagulant. A typical experience is shown in Fig. 5. Over the course of four hours, 4,000 grams of blood were exchanged. Magnesium and potassium were reconstituted in concentrations of 2.5 meq. per liter prior to return of each 500 gram portion of blood to the animal. The last 3,000 grams of the exchange was effected in two hours. This exchange was performed at about the rate of speed of the citrate exchange experiments. There was no specific alteration of the electrocardiogram or arterial pressure contour at the end of the experiment, although four times as much blood had been exchanged as in the citrate experiments. Blood calcium was 9.2 mg. per 100 ml. blood at the start of the transfer and 8.6 mg. at the end of the exchange. Sodium and potassium concentrations showed no significant trend. The maintenance of blood calcium in spite of an exchange of a volume of calcium-depleted blood about equal to the animal's extracellular fluid volume would suggest that the transfer from calcium reservoirs of bone was sufciently rapid to maintain total plasma calcium concentration. Because of the maintenance of total plasma calcium concentration, we cannot ascribe the absence of changes in the physiologic measurements to any isolated cation deficiency. The only suggestive evidence of derangement due to cation deficiency was seen in one animal who developed rapid auricular fibrillation after the exchange of 2,500 grams of blood deficient in both calcium and magnesium. At that time there was somatic muscle tremor, bronchospasm, and dissociation of costal and abdominal ventilatory movement. The fibrillation reverted to sinus tachycardia seventeen minutes after completion of the exchange. Blood calcium dropped from 9.2 mg. per 100 ml. blood to 6.7 mg. per 100 ml. at the time of development of the arrthyhmia and had risen to 8.7 mg. per 100 ml. at the time of reversion to regular rhythm. No significant changes were observed in the QT time or P voltage before or after the period of fibrillation.

Arterial Transfusion of A.C.D.-Treated Blood.—We wondered if the infusion of citrated blood into the proximal arterial system would permit enough direct perfusion of the coronary tree to result in an abnormally high regional concentration of citrate in the myocardium. As shown in Fig. 6, no striking electrocardiographic changes or blood pressure changes occur when A.C.D.-treated blood is perfused at rapid rates directly into the ascending aorta at the level of the aortic valve. The changes noted were equivalent to the changes observed in venous transfusions of citrated blood giving the same cumulative citrate doses at similar infusion rates.

DISCUSSION

The studies presented here indicate that a typical sequential pattern of electrocardiographic change—prolongation of the Q-T interval, depression of the T wave, depression of P-wave voltage, and the appearance of a muscle tremor artifact—develops as massive citrate infusion is carried out with undiluted A.C.D. solution or with A.C.D.-treated blood in artificial kidney experiments or exchange transfusions. This sequence has been described previously

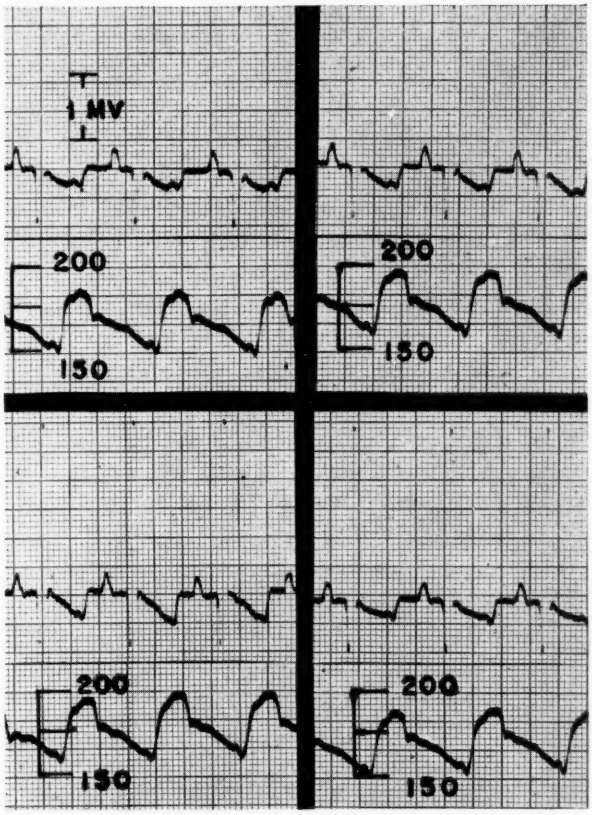

Fig. 6.—Lead II electrocardiogram and aortic arch blood pressure during rapid infusion of A.C.D.-treated blood into the ascending aorta adjacent to the coronary ostia. Prior to this injection, the animal had received three previous injections of citrated blood into the ascending aortic root demonstrating electrocardiographic and pulse contour alterations similar to the changes shown. *Top Left:* Control tracings (0 seconds). *Top Right:* Intra-arterial infusion in progress 60 seconds after start of injection; 99 ml. A.C.D.-treated blood infused. *Bottom Left:* Immediately after interruption of intra-arterial infusion; 215 ml. A.C.D.-treated blood infused in 130 seconds. The citrate introduced during this fourth injection was 3.8 mM total, 0.18 mM per kilogram, 0.083 mM per kilogram per minute. For the four injections, the cumulative dosage of citrate was 16.9 mM or 0.81 mM per kilogram at rapid infusion rates averaging out for the entire period between injections to 0.009 mM per kilogram per minute. *Bottom Right:* 105 seconds after bottom left segment.

in hypocalcemia due to endocrine or renal disease, and has been reported during the exchange transfusion of erythroblastotic infants.[7] The importance of re-emphasizing that such changes can occur, stems from the fact that, in spite of numerous reports concerning the innocuous nature of the citrate contained

in bank blood infused under ordinary circumstances, citrate in large amounts is known to be a toxic agent, and the introduction of new methods of transfusion or the extension of the indications for massive transfusion must be considered in the light of such toxicity.

The finding that rapid infusion of citrated blood may induce early minor variations in the electrocardiogram and in the mode of ventricular ejection is of significance since blood is given at rapid rates for management of clinical conditions in which the functions of cardiac ejection and citrate metabolism may be impaired. Additional study is indicated to determine whether or not side reactions to citrate which may be of clinical significance occur at the time of blood transfusions given at rapid rates and in large volumes in accordance with present-day practice.

Although there is considerable evidence that citrate is rapidly metabolized, certain reports describing rapid metabolic degradation in human beings are based upon the observations of plasma citrate concentration during periods when there may be appreciable dilution of citrate by rapid diffusion out of the plasma compartment. The rapid metabolism of exogenous citrate introduced into the portal circulation suggests that rapid transfer to this area of high metabolic activity does occur. Similar transfers may also take place into regions of less active metabolism than the liver and may account in part for the early rapid disappearance of injected citrate.

Blood exchanged across a resin column, although deficient in several plasma cations, appears to be much less liable than citrated blood to induce electrolyte imbalance. This is probably due to the fact that, in the absence of an excess of ion-binding agent in the injected blood, transfer mechanisms are sufficiently rapid to maintain the concentration of plasma cations in the face of rapid exchange transfusion rates. The maintenance of serum calcium concentration is of particular interest. Further studies are indicated using exchange of resin-treated blood as a method of inducing specific electrolyte disturbance.

SUMMARY

During the course of artificial kidney experiments, derangements of circulatory function were noted. These alterations could be attributed to citrate hypocalcemia induced by the sudden infusion of large volumes of A.C.D.-treated blood used to fill the mechanical dialyzer.

The development of sequential changes in the electrocardiogram, prolongation of the Q-T interval, pulse alteration, depression of the T wave, depression of P-wave voltage, and development of somatic tremor artifact, was observed during the course of infusion of undiluted A.C.D. solution and during the course of exchange transfusion with A.C.D.-treated blood. For constant exchange rates, the electrocardiographic changes occurred at equivalent cumulative citrate doses, but with variations of the rate of citrate infusion, there was an inverse proportion between the rate of infusion and the cumulative dose required to produce circulatory changes. With very rapid

exchange transfusions, small cumulative citrate doses resulted in transient electrocardiographic changes and pulse alternation, which may be of clinical significance in the presence of antecedent myocardial dysfunction.

Blood rendered hypocalcemic by passage across a cation exchange resin did not produce similar circulatory derangements in spite of the exchange transfusion of enormous amounts of blood. Serial analysis of serum calcium levels during the course of such massive exchanges demonstrated that nearly normal concentration could be maintained suggesting that rapid mobilization of calcium could be effected from body reservoirs. This mechanism is probably of importance in sustaining plasma calcium-ion concentration in the face of an elevation of the plasma citrate content.

We are indebted to Dr. John G. Gibson, 2nd, for suggestions during the development of these experiments.

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PLATELET TRANSFUSIONS UTILIZING PLASTIC EQUIPMENT

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INTRODUCTION

RECENT studies have indicated that whole blood can be transfused with measurable survival of blood platelets in thrombocytopenic patients.[1, 2, 3] These results were achieved by the use of polycythemic donors and a direct transfusion technique employing multiple syringes treated with silicone. Blood platelets had a survival period in the recipients of four to six days. Although the need for such transfusions is limited, the procedure may be of great value to control hemorrhage in those patients who have transient depression of platelets. This type of transfusion has limited value in chronic thrombocytopenic states. In such patients, however, platelet transfusions may be helpful in acute bleeding episodes or as an aid to prevent bleeding during surgical procedures. Previous publications have emphasized that platelets may be antigenic.[2, 4] Associated with iso-immunization to transfused platelets, there is a progressive shortening of the survival time of the platelets. This type of sensitization represents a real hazard to the patient and platelet transfusions should be used with caution.

In the past two years the staff of this clinic has had the opportunity to study and use a complete plastic system for the collection and transfusion of whole blood with acid-citrate-dextrose solution as an anticoagulant.[5] In view of the excellent hemorepellent properties of the polyvinyl chloride plastic the present investigation was initiated to determine if a plastic-bag system for platelet transfusion would be efficacious. As experience was gained with whole blood transfusions, efforts were made to determine if platelets could be concentrated in plasma to allow use of normal donors. Polycythemic donors with high platelet counts are not readily available. Such a system would allow easier handling of the blood and exclude breakage of large syringes. Finally, it was anticipated that a plastic system would allow equipment to be available for any hospital staff member to use with ease. A variety of bags and attachments have been tried. The equipment outlined in this paper represents a workable system that utilizes available material so that the cost is minimal. The plastic bag and attachments may be prepared and kept sterile indefinitely. Such a unit can be kept in a blood bank for these transfusions.

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This type of transfusion has been made possible by utilizing an anticoagulant that minimizes the alteration of platelets in shed blood. Dillard and coworkers[6] have shown the particular value of disodium ethylene diamine tetraacetate dihydrate (Na_2EDTA) in preservation of platelets. Platelets do not clump or adhere to surfaces with this anticoagulant. We do not know of any other anticoagulant that will maintain platelets in vitro as observed with Na_2EDTA.

MATERIAL AND METHODS

Patients with thrombocytopenia at the Children's Medical Center and Peter Bent Brigham Hospital were studied. Twenty-four patients were studied and fifty-six platelet transfusions were given. Patients without splenomegaly have been used to evaluate plastic-bag platelet transfusions. Shortened survival of platelets was noted in many instances as has been described previously. In some instances this was anticipated from the nature of the disease; in other patients the shortened survival was attributed to previous blood transfusions. These observations are not listed in this study. Only those patients who showed a four- to six-day survival of transfused platelets have been used to evaluate the preparations of platelets in plastic equipment. A group of patients who received direct transfusions with silicone-treated syringe technique has been previously reported.[2] The results in the patients showing a four- to six-day platelet survival have been used to compare the results obtained with plastic bag equipment.

Platelet Counts and Survival.—Platelet counts were done on capillary blood as previously described.[7] One technician performed all platelet counts. Donor platelet counts were obtained from the plastic bag. Platelet counts of the recipients were done before and at ten minutes, one hour, and three hours after transfusion and daily thereafter.

The theoretical platelet count that could be obtained was calculated:

$$\text{Theoretical platelet count} = \frac{\left(\text{Volume transfused} \times \text{Donor's platelet count}\right) + \left(\text{Patient's blood volume} \times \text{Patient's platelet count}\right)}{\text{Patient's blood volume}}$$

Blood volume was estimated from tables.[8] The percentage of platelets that was observed in the recipient on bases of theoretical platelet count from the transfusion was calculated as follows:

$$\text{Percentage platelet yield} = \frac{\text{Highest posttransfusion platelet count} - \text{Pretransfusion platelet count}}{\text{Theoretical platelet count} - \text{Pretransfusion platelet count}}$$

There was no direct correlation as to what posttransfusion platelet count would be highest. In patients receiving platelet concentrates the three-hour posttransfusion value was usually highest. With whole blood transfusions the ten-minute posttransfusion platelet count was usually highest as had been observed with the silicone-treated technique. The highest posttransfusion platelet count on day of transfusion was used to determine the percentage yield.

To compare various transfusions all platelet counts were calculated on the basis of percentage of platelet yield. Successive daily platelet counts are also expressed as per cent of the initial theoretical yield. Such a procedure allows comparison of individual transfusions. This was done for a three-day period after the transfusion.

Likewise the percentage of platelets remaining each day after transfusion was calculated. The highest platelet count on day of transfusion was used for the 100 per cent value. These values were followed over a four-day period. Such data allowed an estimation of what platelet value may exist to control hemorrhagic diathesis over a period of days.

Donors.—Whenever possible polycythemic donors with high platelet counts have been used for transfusions. Normal donors may be used. One must recognize, however, that large

amounts of whole blood are necessary to elevate platelets above hemorrhagic levels. For example, using estimated blood volume of 5 L. for a 70 kilogram patient, the platelet count can be raised from a level of 10,000 per cubic millimeter to 40 to 50,000 per cubic millimeter with 1 L. of normal blood. This assumes excellent technique with the direct silicone-treated syringe method in which 80 per cent of the platelets would be recovered in the recipient. Two liters of blood would elevate the platelet count to 100 to 110,000 per cubic millimeter. In contrast, 500 ml. of polycythemic blood with a platelet count of 900,000 per cubic millimeter would be quite comparable in the elevation of platelets to that obtained from 2 L. of blood from random normal donors. The large volume of normal blood required is adequate justification for platelet concentration procedures.

Equipment.—In the original study 100 ml. silicone-treated syringes were used.[2] Therefore, it was felt that this quantity of blood was the maximum amount that should be collected before administration to the recipient. A plastic bag* with 125 ml. volume was prepared to equal approximately the amount collected in the large syringes. The plastic bag is made from 0.016-inch-thick transparent polyvinyl chloride 2¾ inch width extruded tubing which is sealed dielectrically at appropriate length. At the center of the top of the bag a 10 inch inlet tube of polyvinyl tubing with an 0.118-inch internal diameter is sealed dielectrically. At the entrance of the inlet tube there is attached a double-ended Nylon Luer adapter. This adapter is shielded with a plastic cover to maintain sterility of the plastic bag unit. The bag is filled with 5 ml. of solution containing 3 per cent Na_2EDTA in 0.85 per cent saline. When these studies were initiated, 12 ml. of a 1 per cent solution was used. However, the 3 per cent solution preserved platelets as well and decreased the volume of anticoagulant. The 3 per cent solution has been used in the majority of transfusions. Later it was observed that larger collections of blood could be made with good recovery of platelets. A plastic bag devised for whole blood collections of 600 ml. has been used in which 30 ml. of the 3 per cent Na_2EDTA is used. To prepare platelet-rich plasma the standard blood bag "Blood-Pack" has been used. This plastic bag also will hold 600 ml. of blood and 30 ml. of the 3 per cent Na_2EDTA solution is used as anticoagulant. All air is excluded from the plastic bag.

All plastic bags have 10 inches of tubing ending with a double-ended Nylon Luer adapter covered by a sterile plastic shield. Contents may be transferred from one bag to another by the use of an adapter coupler.

As noted previously, an arquard-coated[9] 15 gauge male hub Fenwal needle is used.* (Fig. 1). This needle is designed to prevent eddy currents at the attachment of the plastic tubing. The 15 gauge needle will allow rapid flow of blood so that the 600 ml. plastic bag may be filled in eight to twelve minutes. This needle is attached to a 9 inch length of ¼ inch diameter polyvinyl tubing. On the other end of the tubing is attached a flanged plastic "female" adapter. Both the needle and adapter are shielded with plastic covers to keep this unit sterile. Seventeen- and nineteen-gauge Fenwal needles have been coated with arquard and prepared as previously mentioned, when necessary if the recipient has small veins. Standard needles have been coated to use in infants and small children to facilitate venepunctures. In such instances the Luer adapter attachment on the plastic bag tubing is inserted directly into the needle hub.

TECHNIQUE

1. *Direct Transfusion.*—The collection of donor blood is similar to the technique described with silicone syringes. The donor is bled from the antecubital vein. After local infiltration with 1 per cent Novocain, the 15 gauge needle is introduced in the vein and 10 ml. of blood is withdrawn with a syringe to exclude tissue contamination (Fig. 1). The needle tubing is clamped momentarily and the flanged adapter is attached to the double-ended Nylon Luer adapter on the plastic bag outlet. The clamp is removed and the blood is allowed to flow into the plastic bag by gravity. During the period of the phlebotomy the bag is agitated by hand continuously to insure thorough mixing of the anticoagulant. When the

*Manufactured by Fenwal Laboratories, Framingham, Mass.

bag is filled, the tubing is clamped at the needle and bag outlets. The Nylon adapter is disconnected from the needle adapter and the tubing connected with the bag is flushed with a small amount of the 3 per cent Na_2EDTA solution. This is accomplished by an adapter coupler that is attached to a syringe (Fig. 2). The tubing is clamped and a sterile plastic shield is placed over the Nylon adapter.

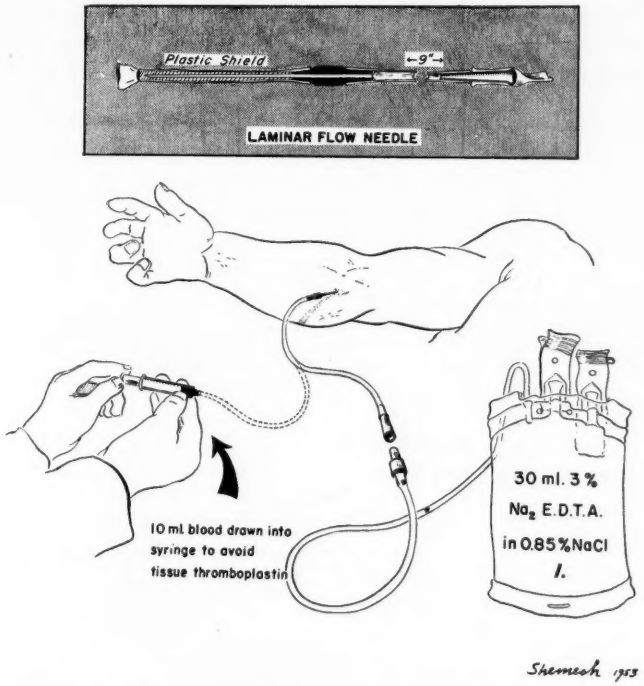
Plastic Shield
←9"→
LAMINAR FLOW NEEDLE
10 mL blood drawn into syringe to avoid tissue thromboplastin
30 ml. 3 % Na₂ E.D.T.A. in 0.85 % NaCl
1.
Shemesh 1953

Fig. 1.—Collection of blood from donor. The laminar flow needle and tubing are covered with plastic shields to maintain sterility.

If the donor is bled near the recipient so that the collected blood can be infused immediately, the flushing of the bag tubing with anticoagulant can be omitted. The recipient is prepared for infusion with the same technique as the donor. After the 15 gauge needle has been properly secured in the antecubital vein, 0.85 per cent saline may be infused until the donor blood is available. The plastic shield is removed from the bag tubing and the Nylon coupler attached to the needle tubing "female" adapter. Previous experience has indicated that fresh whole blood may be infused rapidly. Usually the contents of the bag are given in ten to fifteen minutes. If small children or infants are transfused, a small plastic bag with a volume of 125 ml. is used. Several such small bags may be used to obtain the necessary amount of blood if the volume required is less than 500 milliliters. In some instances the large plastic bag has been used with less anticoagulant and the volume of blood collected is determined by weight as the bag is filled. The plastic bag is squeezed to inject the blood. In some instances the bag has been placed in a pneumatic press for rapid infusion. Adequate results, however, have been achieved by squeezing the bag and rolling it in a tubular fashion to expel the last few milliliters of blood.

Platelet-Rich Plasma.—The blood is collected in the large plastic bag "Blood-Pack" as described. The inlet tubing is knotted after it has been flushed with anticoagulant (Fig. 2). The filled bag is then centrifuged at 1,200 r.p.m. (375 G) for fifteen minutes at 4° C.*

*International Centrifuge Co., Model F.S., Size 3, has been used for this procedure; 6 place centrifuge head with 550 ml. cups is preferable.

This speed will clear the plasma of 80 to 90 per cent of the leukocytes while 75 to 90 per cent of the platelets remain in the plasma. The usual blood bank plasma centrifuge without a refrigerator unit may be used successfully. This temperature may be achieved easily by filling the six large 500 ml. cups with ground dry ice for forty-five minutes beforehand. At that time the cup or cups to be used are emptied of dry ice and filled with ice water to cushion the plastic bag during centrifugation. It is important to surround the bag with water to prevent compression and molding of the bag. The temperature has been maintained at 2 to 4° C. during centrifugation for the fifteen-minute period. No evidence of freezing of the bag or contents has been observed.

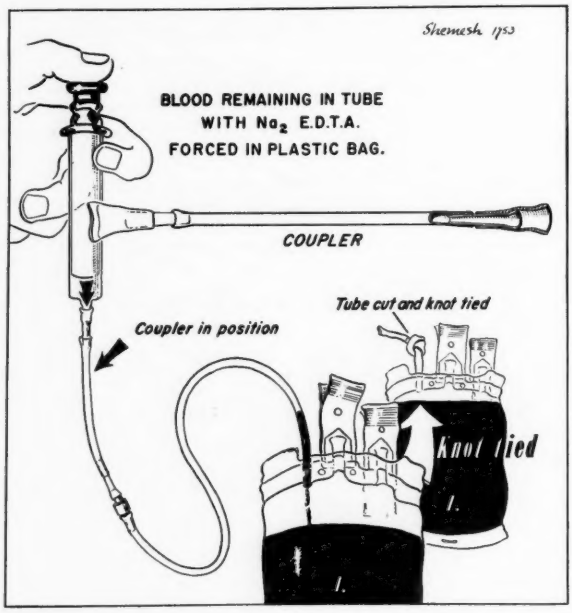

Fig. 2.—Plastic bag tubing flushed with anticoagulant to prevent clotting in inlet of bags. Syringe is attached to the tubing with adapter coupler.

The bag is removed from the centrifuge and placed carefully in a carton or wire basket while the plastic seal at the tip is opened. The protective sheath is removed from a piercing coupler and the cannula is twisted into the delivery tube at the top of the bag to penetrate the seal (Fig. 3). The piercing coupler is attached to the adapter which allows two of the double-ended Luer adapters to be approximated. The plasma is then removed by pressure on the whole blood bag until the blood begins to siphon into the new bag. One may allow the plasma to collect by siphon flow. The flow may be hastened by firm compression over the area of packed erythrocytes. The positive-pressure pneumatic press[5] has been used also with good success. For clinical purposes no effort has been made to exclude white blood cells and a portion of the buffy coat has been removed. Plasma from a liter of blood may be collected in one plastic bag.

The International Centrifuge Co. refrigerated centrifuge, Model PR-1 with 250 ml. cups, may be used with convenience if available. The small plastic bag containing 125 ml. volume is filled as noted above. A donor is bled to fill four small bags. The bags are balanced in the 250 ml. cups with water to cushion the plastic bag. The blood is then centrifuged at 1,750 r.p.m. (375 G) for fifteen minutes. The bags are removed. Inasmuch as some red cells contaminate the inlet tubing, the tubing is clamped close to the bag, washed with antiseptic solution, and cut about three inches from the bag with sterile scissors. The small

shank of a sterile Luer adapter which is shielded over the large end is inserted in the tubing. An adapter coupler then couples the Luer adapter on this tubing to a sterile Nylon adapter on the large plastic bag. The plasma is removed by pressure from each bag.

When the plasma has been harvested a sterile plastic shield is placed over the Nylon adapter of the plasma bag until the plasma is infused. The platelet-rich plasma is infused into the recipient through a 15 gauge needle and tubing as noted for whole blood transfusion. The plasma is chilled in crushed ice until administered.

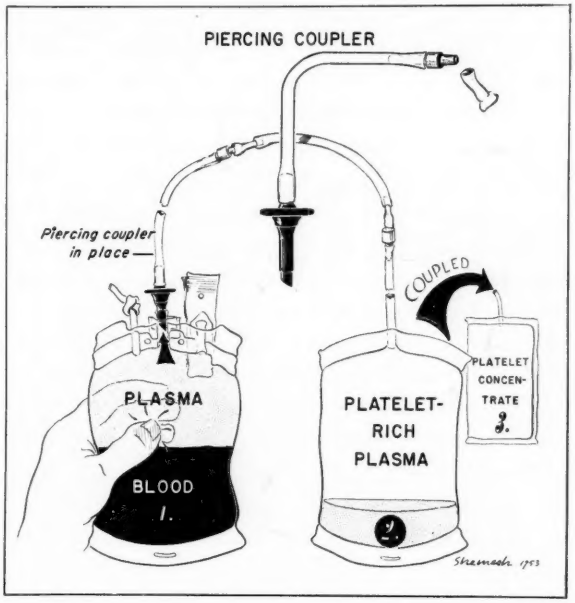

Fig. 3.—Preparation of platelet-rich plasma: A piercing coupler is inserted into plastic bag *1* and connected by an adapter coupler to bag *2*. Plastic bag *2* has a single plastic tube outlet. However, another plastic bag *1* could be used. For preparation of platelet concentrates, the platelet-rich plasma is forced into small plastic bags of 125 ml. volume (*bag 3*) by same coupler attachments. Plastic bag *3* is centrifuged to collect platelets as button in the bottom of the bag.

Platelet Concentrates.—Platelets in plasma may be concentrated by centrifugation of the plastic bag. The platelets and granulocytes will collect as a creamy button at the bottom of the bag. In this study platelet-rich plasma was centrifuged ten minutes at 3,000 r.p.m. (1,000 G) in International Centrifuge Model P.B.I. at 4° C. The large plastic bag cannot be used in this centrifuge. Therefore the platelet-rich plasma was collected in the small 125 ml. bag. The plasma yield from one donor usually will fill two bags. In the four place centrifuge head, plasma from two donors may be concentrated. The bags are cushioned and balanced with ice water as noted previously to hold the bag upright. After centrifugation, the bags are removed and the plasma forced from the bag by positive pressure. Usually 80 to 90 per cent of the plasma is removed. A small aliquot, usually 15 ml. of plasma, is left with the platelet button in the bottom of the bag. The tubing outlet is clamped and each bag is chilled until plasma has been removed from all bags. The platelets in each bag are then resuspended by a gentle rocking motion of the hand over the flat surface of the bag. Firm pressure with the finger will loosen all platelet aggregates from the corners of the bag and allow excellent resuspension. This procedure is done for each bag. If there is more than one plastic bag of concentrate they are attached together by the adapter coupler and the contents of one bag emptied into the other. Each donor will yield 50 to 60 ml. of plasma. Before administration to the recipient, a drop of the cencentrate should be examined to de-

termine if all platelets are discrete. No preparation of aggregates or clumped platelets has been given. In all instances a sample of the whole blood, plasma, or platelet concentrate was obtained for platelet count from the plastic bag before administration to the recipient.

The volume of platelet-rich plasma or concentrate to be transfused is determined by weighing the plastic bag. In this study there has been no correction for specific gravity of plasma or blood. One may anticipate platelet counts in the plasma platelet concentrate of 1 to 5 million platelets per cubic millimeter depending on the initial donor platelet count.

RESULTS

Utilizing the platelet transfusion technique for whole blood described, platelet yields and survivals were similar to those observed with silicone-coated syringes. Fourteen transfusions with silicone-coated syringe technique were evaluated to determine percentage yields and survival of platelets. These transfusions showed a high percentage yield of platelets and four- to six-day survivals. The percentage yield of platelets in the recipients ranged from 55 to 96 per cent with an average of 73 per cent. Some of these transfusions have been previously reported.[2] The results of these transfusions were used to evaluate platelet-rich plasma and platelet concentrate. Eighteen transfusions of whole blood in plastic bags with anticoagulant were studied. This technique was used in patients who were quite ill, and who, in many instances, had shortened platelet survival by silicone syringe technique. Only five studies showed good survival over a four-day period and were used for evaluation. Platelet yields in the recipient varied from 50 to 95 per cent with an average of 74 per cent for the five procedures evaluated. These results would appear to be quite comparable to the silicone-treated syringe technique. Hence, these values were not charted.

Ten transfusions of platelet-rich plasma were selected from 22 procedures. By inspection of the individual survival periods, ten transfusions showed an average survival of four to six days. The other transfusions had shortened platelet survival in patients in whom this also had been observed by whole blood platelet transfusions. The mean percentage yield of platelets was 66 per cent and varied from 35 to 91 per cent in individual transfusions. One may note in Fig. 4 that the percentage yields from day to day are comparable to those observed with silicon syringe transfusions.

Platelet concentrate plasma transfusions were used in 16 procedures. Ten of these transfusions showed normal survival periods and were used to evaluate this technique. The initial recovery of platelets in the recipient was less with a mean percentage yield of 52 per cent. Individual percentage yields varied from 36 to 81 per cent. Platelet concentrate plasma had the poorest yield in the recipients. Although the percentage yield of platelets remained similar throughout the three-day period charted, the discrepancy noted during the posttransfusion periods was markedly lessened after twenty-four hours (Fig. 4).

It was of interest to determine the comparative number of platelets that would persist in the recipient after each transfusion. The platelet count each day after transfusion was compared with the highest platelet count post trans-

fusion and expressed as the remaining percentage of transfused platelets (Fig. 5). There was a marked variation in the decline of the remaining platelets among the three methods. On inspection of individual transfusions this variation was not correlated with the elevation of the platelet count. The platelets from silicone-coated syringe and plastic bag whole blood transfusion disappeared most rapidly during the first twenty-four hours and 63 per cent of the platelets remained after twenty-four hours. The recipients receiving platelet-concentrate plasma maintained a higher percentage of platelets, 86 per cent. Platelet-rich plasma was intermediate in the number of surviving platelets

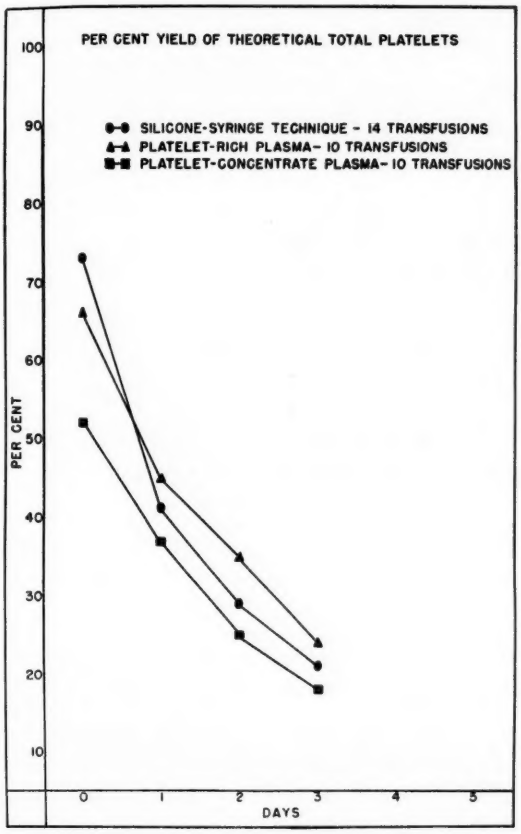

Fig. 4.—Mean percentage yields of platelet transfusions in thrombocytopenic recipients by techniques described. Silicone-coated syringe transfusions yielded highest platelet percentages in recipients.

during the first twenty-four-hour period, 75 per cent. Within forty-eight hours the platelet survival percentages were approximately alike in all three procedures and maintained such similarity during the period that survival was followed.

DISCUSSION

No detailed statistical evaluation of the data has been made. There is at least a possible 10 per cent error in the blood volume estimations used for these calculations.[8] Hence, only arithmetical means of the various transfusion

procedures were used to compile the platelet yields during the period of time observed. The associated errors of platelet counting also contribute to the problems of evaluation. Blood volume measurements with dyes or tagged proteins would be of value in future studies.

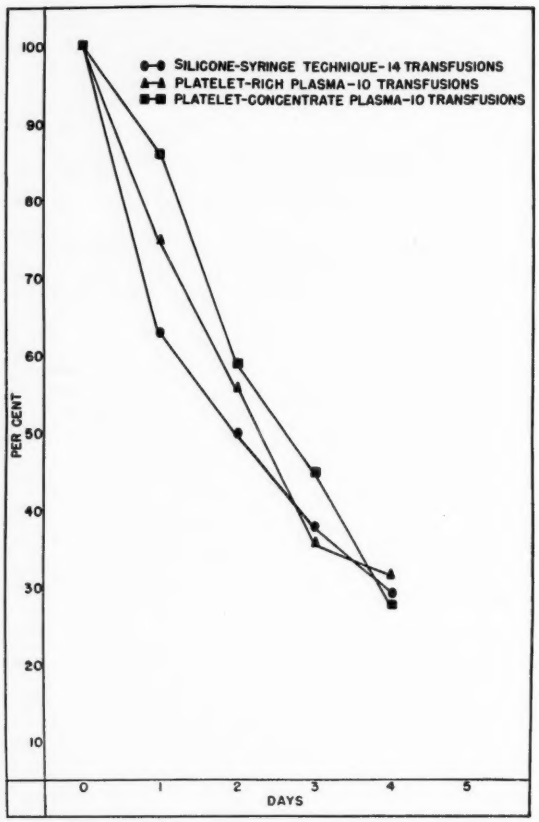

Fig. 5.—Comparative percentage values of surviving platelets. The number of platelets remaining each day was compared with the highest posttransfusion platelet count. A larger number of platelets survive in the recipient after platelet concentrate transfusions during the first twenty-four-hour period.

The direct transfusion of whole blood by silicone-coated syringes or plastic bags with anticoagulants gave the highest initial yield of platelets in the recipient. Platelet-rich plasma requires forty-five to sixty minutes to prepare for infusion. There is a slight loss of average yield despite all efforts to limit time during the procedure. The platelets that do survive these manipulations have survivals similar to those of whole blood procedures.

Platelet concentrates have a longer delay in preparation before transfusion. Usually one and a half hours have been required. Platelet yields by this method might have been better if the time interval had been shortened. In a few instances transfusion of small volumes to children has shortened the

time interval for preparation of platelet-rich plasma or platelet concentrates. Similar platelet yields were obtained by three different procedures in the recipient where small aliquots of blood and plasma were used (Fig. 6). No studies were done to determine viability and survival of platelets during varying time intervals after collection of blood. The factors involved in the smaller percentage yields of plasma and plasma concentrates cannot be defined completely. Hirsch and co-workers[10] have demonstrated the marked metabolic activity of platelets. Hence, every effort was made to chill the blood and plasma during these procedures to inhibit such activity. Inasmuch as information is not complete regarding metabolism of platelets, certain nutritional

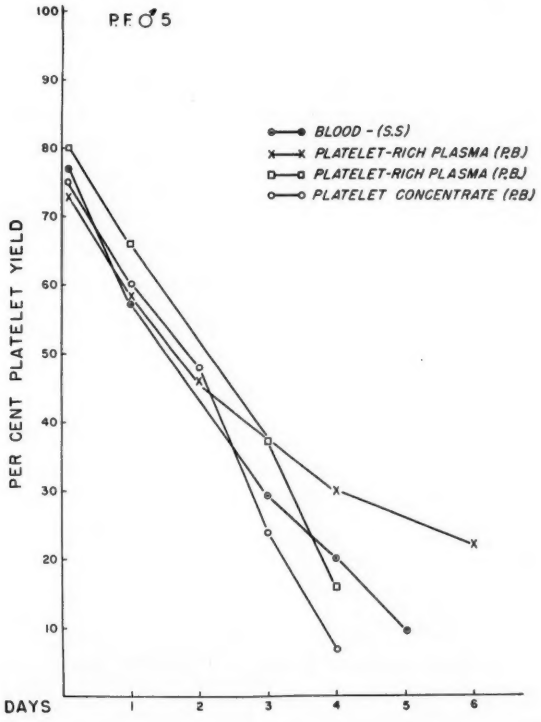

Fig. 6.—Comparison of per cent theoretical platelet yields in same thrombocytopenic recipient; 100 ml. polycythemic blood given by silicone-coated syringe technique (S.S.); 100 ml. of platelet-rich plasma in plastic bags (P.B.) given twice; 42 ml. of platelet concentrate given in plastic bag (P. B.).

components also may be deficient during the period of preparation. Finally, little information was obtained regarding the effects of packing platelets in the plastic bags. Although the platelets resuspended completely after centrifugation from the creamy button formed in the bottom of the bag, one may suspect that this procedure has altered their surface. On microscopic examination the platelets had spicule formation, but this did not appear different to any extent from platelets maintained in plasma for this additional period of time

without centrifugation. We have assumed that the time interval and platelet packing may explain the initial lower yield of plasma concentrates in the recipient.

It is of interest to note that a higher percentage of platelets are retained in recipients receiving platelet-rich plasma or platelets concentrated during the first twenty-four-hour period after transfusion (Fig. 5). No adequate explanation is available for these observations. Platelet-rich plasma and concentrates have been examined microscopically to determine whether the platelets exist in aggregates after centrifugation. These studies have been done with silicone-treated slides and cover slips. The weight of the cover slip, however, may disperse aggregates of platelets which exist in the plasma. These preparations were not studied by hanging-drop technique to determine if aggregation did exist after a period of time. In such an event, the mixing of the platelets with the total circulating blood volume may be delayed because of transient sequestration of such platelet aggregates. Also dispersion of platelets intermixed with red blood cells may be an aid for more complete mixing of the platelets when whole blood is given. Finally, physical alteration of the platelet membrane during centrifugation and packing may have altered mixing in the circulation. The higher platelet percentage retained for the first day after transfusion with platelet concentrates suggests that this procedure may be of greater therapeutic value, inasmuch as less volume need be given to the recipient with assurance of higher platelet levels during the initial post-transfusion period.

SUMMARY

A procedure has been outlined for the use of plastic equipment for platelet transfusion. Disodium ethylene diamine tetracetate dihydrate has been used as an anticoagulant in preparation of platelet-rich plasma and platelet concentrates. Smaller percentage yields of platelets were observed in the recipients when platelet-rich plasma and platelet concentrates were transfused. The smaller volume required when plasma rather than whole blood is infused permits the use of normal donors for platelet transfusions instead of polycythemic donors with high platelet counts. The results of this technique have been compared with direct platelet transfusion utilizing silicone-coated syringes with respect to percentage yield and survival of platelets.

We are indebted to Dr. Carl Walter and Louis K. Diamond for their aid in this study.

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STUDIES ON THE NORMAL SERUM PANAGGLUTININ ACTIVE AGAINST TRYPSINATED HUMAN ERYTHROCYTES

III. Panagglutinin Titers in Infants and Young Children

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PREVIOUS studies have shown that an agglutinin could be found in all of a large number of normal human serum tested, which was capable of agglutinating trypsinated erythrocytes of the same blood specimen.[1, 2] Some of the properties and reactions of this normal auto-agglutinin have been described.[2, 3] It has been found to be distinct from cold agglutinin, to be mainly concentrated in Cohn's fraction I + III, and is capable of being absorbed and subsequently eluted from trypsinated erythrocytes. Inasmuch as this normal auto-agglutinin (NAA) has certain properties in common with antibodies, studies were undertaken to determine whether the development of the NAA titer with age showed a corresponding similarity to that of other known antibodies. In the studies to be reported the development of cold agglutinin titers was compared to that of NAA titer in infancy and early childhood.

METHODS AND MATERIALS

The subjects used in the present study were healthy patients from the well-baby clinic, and unselected newborn infants whose blood was tested about five days after birth. Whole blood was collected in clean glass tubes and allowed to clot at room temperature. When sufficient clot retraction had occurred, serum was separated by centrifugation, and red blood cells were collected by breaking the clot in saline with an applicator stick. The cells were washed and trypsinated according to techniques previously described.[3] All agglutination studies were performed with the serum of the subject tested against his respective cells, i.e., only auto-agglutination was studied. NAA titers were determined by serial dilution method in which agglutination was read immediately after the trypsinated erythrocytes and the appropriate serum dilution had been mixed and promptly centrifuged.[1, 2] It is important that these tests be read without delay, as agglutination reversal occurs in such tests as a constant phenomenon within thirty minutes or less.[1, 2] Cold agglutinin titers were determined against trypsinated erythrocytes by incubating appropriate mixtures of cells and serum for sixteen hours at 4° C. Agglutination was read without centrifugation.

RESULTS

Fig. 1 shows the development of the NAA titer with age as compared to that of cold agglutinin titer against trypsinated cells. It is to be noted that the NAA activity of newborn infants as well as that found in the various age groups studied is quite constant. At no age level was there an appreciable difference in NAA titer as compared to those in the adult series previously reported.[3] Of further interest is the finding that agglutination reversal proceeds quite com-

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parably in the present series of subjects to that found in adults. In contrast, the pattern of cold agglutinin titer shows a distinct developmental trend. Cold agglutination is found in none of the subjects under six months of age, and then begins to appear in low titer in an occasional subject of the next few age periods. There is a progressive tendency to increased frequency and activity to the age of about fifteen months, when the distribution approaches that seen in adults. In no case could the development of a cold agglutinin in any particular subject be traced to a definite infection or illness.

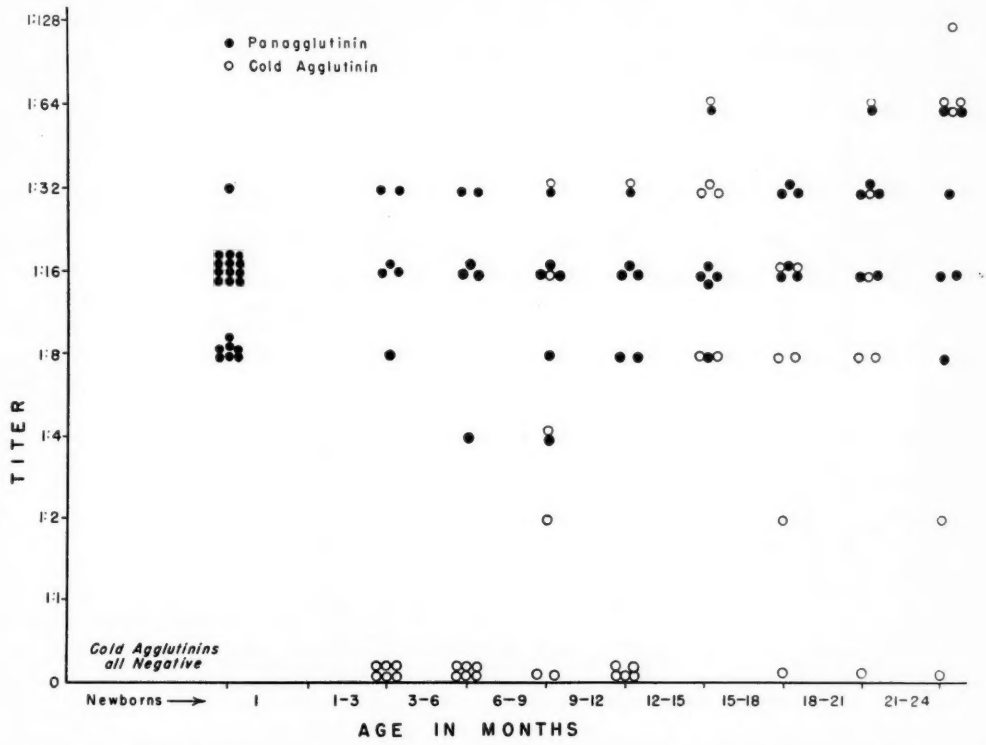

Fig. 1.—Effect of age on NAA and cold agglutinin titers. Each dot represents one subject.

DISCUSSION

The present studies indicate that the human auto-agglutinin active against trypsinated red blood cells is an agent which is present irrespective of either developmental or environmental influences, at least in the postnatal period. This finding is germane to the question as to the antibody nature of NAA. The classical definition of antibody is ". . . a humoral globulin produced by the body in response to an antigen, and capable of reacting with the antigen in some observable way."[4] NAA produces a typical agglutination reaction with appropriately treated cells, can be demonstrated on their surface, and can be eluted off them with suitable techniques. In these respects its behaviour resembles that of many other true antibodies. In contrast, the failure to show

a developmental pattern with growth suggests that NAA does not form in response to specific antigenic stimuli. Although the newborn infant may show an appreciable antibody content in its globulins as a result of maternal transfer across the placental barrier, this typically is largely lost in the first few weeks of life. Subsequent replacement of antibodies in infant blood occurs when the antibody synthesis mechanisms mature sufficiently. The anti-A and anti-B isoagglutinins show a maturational development similar to that of antibodies (Fig. 2) and thus seem to behave as true antibodies. Likewise, cold agglutinins do not appear until an age when the ability to produce known antibodies becomes manifest. The failure of age to influence NAA titer strongly suggests that this substance is not an antibody as defined above.

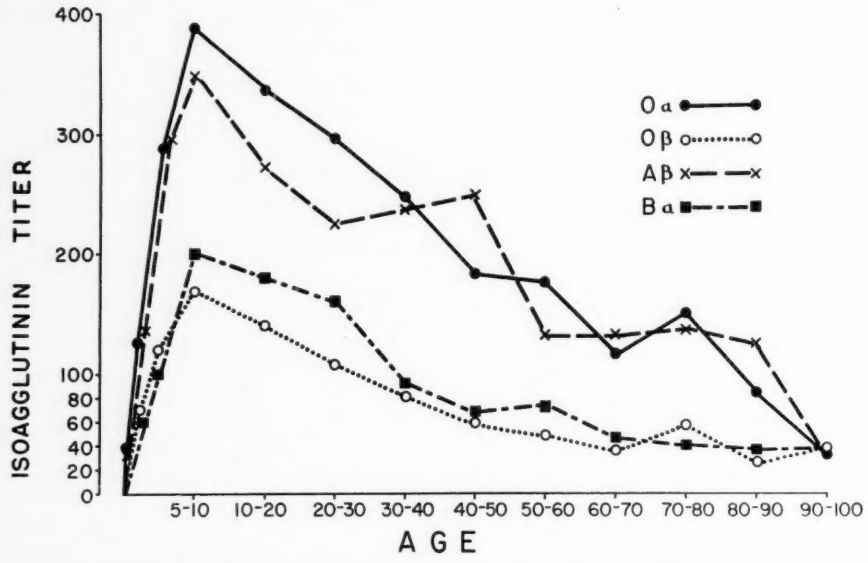

Fig. 2.—Influence of age on normal iso-agglutinins. (From Thomsen, O., and Kettel, K.: Ztschr. f. Immunitätsforsch. u. exper. Therap. 63: 67, 1929.)

It would thus appear that NAA represents the incidental activity of some serum protein component, the function of which may well be found in some entirely unrelated activity. Whether this activity has any physiologic significance is entirely unknown. The presence, however, in human blood of an antibody-like substance not appearing in response to antigenic stimulation may be of significance. It is common practice these days to speak of certain disorders as being associated with auto-antibodies. In acquired hemolytic anemia and in idiopathic thrombocytopenic purpura circulating substances have been found which behave toward erythrocytes and platelets, respectively, in a manner simulating that of established antibodies.[5-8] These findings have led to the conclusion that an abnormal immune mechanism is at work. It is entirely possible that in these diseases the alternative explanation is correct; that the circulating hemagglutinating agents represent a nonspecific activity of some abnormal protein.

SUMMARY

The activity of the normal serum agglutinin active against trypsinated erythrocytes (NAA) was studied in infancy and early childhood and compared to cold agglutinin titers in the same subjects. There was no appreciable difference between NAA titers of infants and adults, nor was any influence of age detected. In contrast, cold agglutinins were absent in infancy and appeared after the age of six months. These findings suggest that NAA is not an antibody.

We wish to acknowledge the kind cooperation of Drs. Muriel Bennett and Herman Lipow from the Department of Pediatrics, Stanford University, School of Medicine.

Addendum: Since this manuscript was submitted for publication, Hoyt and Zwicker[9] have reported studies in which the interesting suggestion is made that NAA represents anti-trypsin activity. Although their evidence for this view is indirect, the hypothesis is readily subject to experimental verification. This is necessary before NAA and serum anti-trypsin can be considered identical.

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MEASUREMENT OF ERYTHROCYTIC DIAMETERS WITH PHASE MICROSCOPY

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EXACT determination of the diameters of erythrocytes suspended in serum or plasma is impossible with the ordinary microscope and standard bright-field illumination, except by adoption of complicated procedures which are time consuming and which hinder simultaneous studies of the cells from other aspects.[6] This is due to the interference offered to sharp optical definition of the margins of the cells by peripheral reflections and diffraction bands (Fig. 1). These are intensified by the incessant rhythmical[2] and Brownian movements of erythrocytes in plasma. While there is less interference to sharp definition with fixed erythrocytes, the diameters of the cells, on the other hand, are shrunken to various degrees by fixation, and measurements of them are misleading.[5] The mean diameters of unfixed and fixed human erythrocytes were found to be 8.8 and 7.6-7.0 microns, respectively, by Ponder and Millar,[4] and 8.5 and 7.6-7.1 microns, respectively, by Ponder and Saslow.[5]

In contrast to the status with the standard microscope and bright-field illumination, the edges of unfixed erythrocytes appear sharp and thin with the phase microscope (Fig. 2) and permit careful definition, even with initial magnifications that are considerably greater than those usually with the ordinary microscope and bright-field illumination. The sharpness of outline and increase in magnification, in turn, permit use of calibrated ocular scales and direct readings of the diameters. While obvious, the Brownian and rhythmical movements of the cells no longer present difficulties.

MATERIAL AND METHOD

The measurements in this report were made upon the erythrocytes of three normal women. M. A. G. is of Italian extraction and has numerous oval cells in circulation which had to be omitted from the measurements made in obtaining the mean diameters. The blood was drawn by deep puncture of a finger. It was either touched directly to a cover glass, inverted upon a microscopic slide, allowed to film by capillarity, and rimmed with petroleum jelly to prevent evaporation; or it was allowed to clot in a tube first, from which drops in serum were filmed in the same manner. No form of staining was used.

Furchgott[3] demonstrated the effects of pH, distances between opposing surfaces and quality of the surfaces upon the sphering of washed erythrocytes. Hydrogen ion was not a problem in the present measurements, since they were made in plasma or serum, but a buffering agent is advisable when the measurements are to be made in other media. Locke's solution was found to be sufficiently buffered for our measurements made in saline solutions of bovine crystalline albumin.[7] Time must be allowed for readjustment when

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the cells are suspended in foreign media, and the intervals between measurements so spaced as to indicate if the cells have come to equilibrium. The slides and cover glasses were cleaned as for supravital preparations.[1] Number 0 cover slips were used.

Films were used for measurement only when the thinner areas were evenly spread and were free of rouleaux formation, overlapping of cells, or evidences of flow or of pressure between cells, and yet which allowed the free amoeboid movements of leukocytes which indicate that there is adequate capillary space beneath the cover glass to prevent compression of the erythrocytes. The films were examined with a Spencer phase microscope, using bright, medium-phase contrast, i.e., erythrocytes appearing moderately bright and moderately flat against a darker background. An oil immersion objective and 15 × ocular were used in conjunction with an ocular scale, the smallest division of which was equivalent to 0.53 micron under this magnification. The field was slowly moved from left to right, to left, as for a differential count. Those cells were chosen for measurement which appeared normally disc-shaped, translucent, round, and without surface irregularities, which were not touched by other cells, and in which both ends of the diameter could be brought simultaneously into clear focus with the scale (center cell, Fig. 2). They were chosen in sequence as they passed across the scale and with effort to avoid influence in selection based on measurements already made. Ten such measurements were made upon each of two separate films for each set of studies, giving a total of twenty cells measured per individual per study. The many additional measurements to the ones reported here which were made upon the erythrocytes of these individuals in the course of investigation of the effects of albumin upon erythrocytic diameters[7] serve to substantiate the dependability of these procedures.

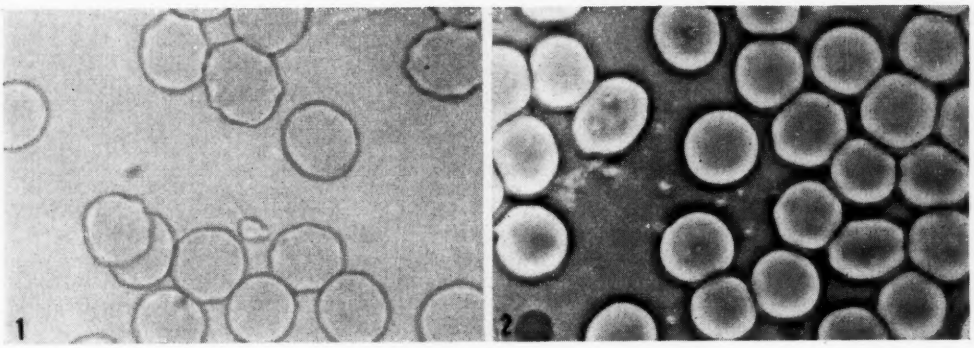

Fig. 1.—Supravital film of human blood (without stain). Viewed with the ordinary microscope and standard, bright-field illumination. Exact determination of the margins of the cells is impossible, due to peripheral reflections and diffraction rings. (2 mm. oil immersion objective [n.a. 1.4], and 15 × ocular.) (×1,350; reduced ⅛.)

Fig. 2.—Similar film to that for Fig. 1. Viewed with bright, medium-phase contrast microscopy. The edges of the cells appear thin and sharply defined. Measurement can easily be made by use of an ocular scale. (97 × bright, medium-phase contrast oil immersion objective, and 15 × ocular.) (×1,455; reduced ⅛.)

RESULTS

The mean of the twenty measurements per study is accepted as the average diameter of the erythrocytes of the individual at the time of measurement. Table I presents the mean erythrocytic diameters under normal conditions of the three individuals under study, together with the statistical data needed for evaluation. It will be seen that the deviation from the mean in any given set of observations is small and that, with the exception of the first set of measurements of the erythrocytes of M. A. G., the means for the three individuals under study are in relatively close agreement with each other and with the values ob-

tained by Ponder and Millar[4] and Ponder and Saslow[5] for unfixed human erythrocytes by photographic procedures. These determinations were made easily and relatively quickly in the course of other studies upon the same cells.[7] It is this ease and speed of measurement, actually, and the fact that measurement can be carried out simultaneously with other studies upon viable erythrocytes, that make the method especially useful.

CONCLUSIONS

Phase microscopy facilitates direct measurement of the diameters of unfixed erythrocytes. The figures obtained for the mean diameters of the erythrocytes of three normal females are in accord with the values reported for human erythrocytes by photographic procedures. The variations around the mean of any individual set of observations are small.

TABLE I. MEASUREMENTS OF ERYTHROCYTIC DIAMETERS*

DONOR	DATE	MEAN DIAMETER μ	DIAMETERS IN SMALLEST DIVISION OF OCULAR SCALE (ONE SMALLEST DIVISION EQUIVALENT TO 0.53 μ)		
			MEAN OF 20 MEASUREMENTS	STANDARD DEVIATION	DEVIATION OF MEAN
E. H. T.	12/ 8/50	8.9	16.7	0.51	0.12
	1/30/51	8.8	16.6	0.48	0.11
M. A. G.†	12/ 4/50	8.3	15.6	0.65	0.15
	1/31/51	8.9	16.8	0.62	0.14
A. M. G.	12/ 6/50	8.9	16.7	0.90	0.21

*We are grateful to Miss Olive Lombard for her aid in this statistical analysis.

†The difference between the means of the two series of measurements on the cells of M. A. G. is obviously due either to differences in the biologic state of the cells at the two times of measurement, or to some unrecognized modification of optical technique, since the deviations between the individual measurements in these two series do not differ from those obtained in the rest of the series.

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ARTIFACTUAL DIFFERENCES IN THE DISTRIBUTION OF T-1824 AND I^{131}-LABELED ALBUMIN RESULTING FROM MIXING PRIOR TO ADMINISTRATION

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STUDIES in this laboratory of the comparative immediate distribution of T-1824 and I^{131}-labeled human serum albumin (I.H.S.A.) necessitated the mixing of these agents prior to their administration.[1] Others have similarly combined T-1824 and macromolecular antigens in a single injection mixture in order to contrast the relative dilutions of dye and foreign protein.[2] Although theoretically desirable, such preparations of "single shot" mixtures may alter the physicochemical properties of one or all of the component indicators. Thus, unless dye is present in marked excess, the available protein in the mixture may bind all of the T-1824 in vitro and thereafter preclude in vivo evaluation of anything save the fate of a T-1824-protein complex. Attempts were made to minimize any prebinding in our studies by utilizing a labeled protein of high specific activity and combining this with dye in proportions designed to bind maximally no more than 5 per cent of the T-1824 at pH 7.4. It soon became apparent that addition of a preponderance of dye was not without effect upon the protein moiety. The combination rendered a fraction of the iodinated albumin disproportionately vulnerable to early removal from the blood stream. Thereafter, the apparent dilution of radioactivity exceeded that of dye and the ratio of $\frac{\text{"T-1824 space"}}{\text{"I.H.S.A. space"}}$ ranged from 0.72 to 0.96.

Such early disparate distribution of T-1824 and iodoalbumin was not observed when the same indicators were given by simultaneous but separate administration.

Experiments were designed to investigate the biologic and chemical behavior of iodoalbumin combined with large amounts of T-1824 in vitro.

MATERIALS AND METHODS

I^{131}-labeled human serum albumin which incorporated iodine with albumin at a 2:1 molar ratio was obtained from a commercial source.* The quoted specific activity of this preparation was "300-700 μc of I^{131} per five milligrams of albumin in each milliliter of neutral, approximately isotonic solution." The same preparations have been used by other investigators in many published studies.[3-6] When each lot of I.H.S.A. was received

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the radioactivity was reassayed. When sufficient material was available, the protein concentration of the solution of iodoalbumin was verified by the modified biuret method of Reinhold, Stewart, and Gilman[7] or by estimation of its nitrogen content by a modified micro-Kjeldahl technique. Homogeneity of the albumin was appraised for every third lot by electrophoresis or ultracentrifugation. Residual inorganic I^{131} was measured after dialysis for twenty-four to one hundred twenty-four hours against distilled water and against 5 per cent potassium iodide. Contamination with unbound iodide was also estimated by precipitating carrier-enriched aqueous solutions of I.H.S.A. with trichloracetic acid.

Ampuled 0.5 per cent aqueous solutions of T-1824* diluted with sterile distilled water were used for most of the studies. Weighed amounts of powdered T-1824† were employed for the experiments with dogs Nos. 11 to 14. In the latter preparation, a trace of red impurity could be demonstrated by ascending paper chromatography with a butanol-acetic acid solvent system.

Optical densities of dyed solutions were determined at 620 mμ in a Coleman Jr. spectrophotometer set for 100 per cent transmittance with a comparably diluted reference standard. Plasma specimens for spectrophotometry were centrifuged at least twice for fifteen minutes at 3,000 r.p.m. Hemolyzed or turbid specimens were discarded.

Radioactivity was measured with a thin mica-end window Geiger-Müller counter. Duplicate planchets were counted sufficiently long to reduce the standard deviation of the count to less than 1 per cent. Correction for self-absorption was obviated in acute experiments by comparable dilution of all specimens. In prolonged studies, all samples were weighed and a correction was made for self-absorption by means of a mass-absorption curve.

Ultracentrifugal studies were performed in the Spinco analytical centrifuge at 47,000 r.p.m. Sedimentation constants of various preparations of human serum albumin were determined by the refractive index method. Electrophoretic analyses were performed in a Perkin-Elmer Electrophoresis apparatus, using the schlieren scanning system.

Mixtures of dye and labeled albumin were prepared. Aliquots from various lots of I.H.S.A. were added to aqueous solutions of T-1824 in amounts calculated to prebind no more than 5 per cent of the available dye anions. Relative proportions were based on a theoretical maximal union of 11 moles of T-1824 (M.W. 960) with one mole of albumin (M.W. 70,000) at pH 7.4.[8, 9] In practice more sites may have been available for dye-linkage since the mixtures approximated the pH of the diluted aqueous T-1824. The final ratio of dye to protein could be precisely predicted where sufficient amounts of the commercial I.H.S.A. had been available for preliminary measurement of its protein content. In the remaining instances, the manufacturer's estimated concentration of protein in the undiluted I.H.S.A. (i.e., 500 mg. per cent) was employed to derive approximate molar relationships. Cellophane strips immersed in these mixtures became discolored, thereby substantiating the presence of unbound dye anions.[8] The effects of T-1824 in marked excess upon iodoalbumin were assessed by studying the behavior of these mixtures both in vivo and in vitro.

EXPERIMENTAL PROCEDURES AND RESULTS

1. *In Vivo Studies.*—Subjects were convalescent hospital patients and laboratory personnel between the ages of 18 and 35. Premedication consisted of 20 drops of Lugol's solution given on the evening preceding the experiments. Observations were also secured in mongrel dogs which had been lightly anesthetized with Nembutal. In all studies, the subjects were recumbent and had fasted for at least twelve hours.

*William R. Warner Co., New York.
†Eastman Kodak Co., Rochester, N. Y.

Subjects were given 10 to 25 mgm. of T-1824 and 0.40 to 3.0 mgm. of albumin labeled with 20 to 60 μc. of I^{131}. These indicators were delivered in a single injection mixture or by separate, simultaneous administration of equal volumes.

Blood samples were drawn without stasis from indwelling retention needles in the femoral artery or antecubital vein. Specimens were delivered into silicone-coated test tubes containing 1 mg. of dried heparin.

Nineteen subjects were given a mixture of T-1824 and I.H.S.A. by single injection. Bloods were drawn at five-minute intervals during the first half-hour and the slopes of disappearance of dye and radioactivity during this period were extrapolated to zero time. The compartments available for the distribution of T-1824 and I.H.S.A. during the first half-hour (i.e., "the rapidly exchanging T-1824 or I.H.S.A. spaces"[10]) were derived by dividing the amount of each indicator which had been administered by its concentration at zero time. Dilution of I.H.S.A. exceeded that of the concurrently administered T-1824 in every instance. Ratios of "T-1824 space/I.H.S.A." space averaged 0.898 (Table I).

The phenomenon also could be demonstrated in dogs given a single mixture of iodoalbumin combined with a large excess of T-1824 (Table I). When similar amounts and even identical preparations of dye and I.H.S.A. were administered simultaneously but by separate administration into contralateral veins, no such disparate distribution could be demonstrated. Dilutions of dye and protein were identical and T-1824/I.H.S.A. ratios approximated unity, as others have reported (Table I).[6, 11]

Although the prior addition of marked excess of T-1824 altered the distribution of I.H.S.A. during the first half hour, the rates of disappearance of dye and radioactivity from the bloodstream were identical after the first ten minutes. It was inferred that the enhanced dilution of dyed iodoalbumin resulted from an early disproportionate destruction or sequestration of a heterogeneous component of the I.H.S.A. Therefore, in six subjects, blood specimens were collected at intervals of thirty to sixty seconds during the first ten minutes following administration of dye-protein mixtures into the subclavian vein or peripheral arteries. Loss of iodoalbumin in excess of T-1824 could be uniformly demonstrated after the first few circulations (Figs. 1 and 2).

Four to six hours following administration of T-1824 and I.H.S.A. by separate or combined injection, no significant amounts of thyroidal radioactivity could be demonstrated with a directional Geiger-Müller counter. Since all subjects had been pretreated with Lugol's solution, it was assumed that urinary excretion of I^{131} would more adequately reflect variations in the metabolic breakdown of dyed and undyed I.H.S.A. Urinary excretion of radioiodine, however, during the first twenty-four hours did not differ significantly among seven subjects given I.H.S.A. alone (mean 14.8 per cent of the administered radioactivity; S.E.M. $\pm$ 1.13 per cent) and 18 subjects given mixtures of T-1824 and I.H.S.A. (mean 15.7 per cent; S.E.M. $\pm$ 1.09 per cent). Both values for

TABLE I. EFFECT OF TECHNIQUE OF ADMINISTRATION ON THE INITIAL DISTRIBUTION OF I.H.S.A.

PATIENT FILE NO.	WEIGHT	DIAGNOSIS	I.H.S.A. LOT NO.	DISTRIBUTION COMPARTMENT T-1824 (ML.)	I.H.S.A. (ML.)	T-1824 / I.H.S.A.
		Combined Injection of Excess T-1824 Mixed With I^{131}-Albumin				
5	76.8	Thrombophlebitis, left leg	1	2280	2368	0.963
32	90.6	Peptic ulcer	1	3386	3526	0.960
33	69.3	P.O.* myxosarcoma removal	1	3267	3392	0.963
4	69.5	Peptic ulcer	2	2566	3047	0.842
7	71.1	Frost-bite	3	2870	3176	0.904
34	75.0	P.O.* removal plantar fibroma	3	2326	2591	0.898
35	60.5	Hepatitis	3	2348	2582	0.909
8	53.2	P.O.* subtotal gastrectomy	3	2303	2632	0.875
36	67.7	Bronchial asthma	3	2854	3093	0.923
37	75.0	A-V fistula	4	2693	2950	0.913
38	79.5	A-V fistula	4	3230	3460	0.934
39	81.8	P.O.* removal shell fragment	5	2775	3205	0.866
44	62.7	Peptic ulcer	6	2794	3164	0.883
45	66.4	Polycythemia	7	3281	3804	0.863
46	78.6	A-V fistula	8	3106	3473	0.894
40	65.5	A-V fistula	9	2748	2972	0.925
56	68.9	Laboratory worker	10	2360	2739	0.865
57	96.8	Laboratory worker	10	4090	4626	0.882
58	75.9	Laboratory worker	10	3050	3626	0.841
	Mean					0.898
	Standard deviation					0.045
	Standard deviation of the mean					0.010
Dog No. 1	9.1	----------	14	249	348	0.716
Dog No. 2	10.0	----------	15	469	550	0.853
Dog No. 3	11.4	----------	15	516	613	0.842
		Separate and Simultaneous Injection of T-1824 and I^{131}-Albumin				
59	73.8	P.O.* A-V fistulectomy	13	2722	2764	0.985
60	48.6	P.O.* A-V fistulectomy	13	2437	2451	0.994
61	66.6	P.O.* A-V fistulectomy	13	3152	3164	0.996
62	59.2	P.O.* A-V fistulectomy	13	2366	2395	0.988
63	70.0	P.O.* A-V fistulectomy	13	2508	2469	1.016
48	75.0	Laboratory worker	11	2941	2655	1.108
49	63.6	Laboratory worker	11	2342	2337	1.002
51	74.5	Laboratory worker	11	2542	2454	1.036
6	71.8	Laboratory worker	12	a) 2945	2841	1.037
				b) 2811	2866	0.981
50	70.7	Laboratory worker	10	2551	2559	0.997
47	85.5	Laboratory worker	10	3239	3225	1.004
	Mean					1.012
	Standard deviation					0.035
	Standard deviation of the mean					0.010
Dog No. 7	10.0	----------	16	444	432	1.027

*Postoperative.

urinary losses are high and compatible with recent reports of biologically heterogeneous components in some lots of commercial iodoalbumin.[12] In a single subject (No. 56), only 0.11 per cent of the injected radioactivity could be recovered in catheterized urine obtained thirty-minutes after injection of a mixture of I.H.S.A. and T-1824, despite the fact that all of the disparate loss of iodoalbumin had occurred during the first seven minutes. Similarly, all

attempts to demonstrate significant amounts of radioactivity within the red cells or in plasma dialysates during the first thirty minutes were unsuccessful.

Rawson[8] and Allen and Orahovats[9] have demonstrated that a single molecule of albumin can maximally bind no more than 11 to 14 moles of dye at pH 7.4. All disparities in distribution, reported previously were observed when

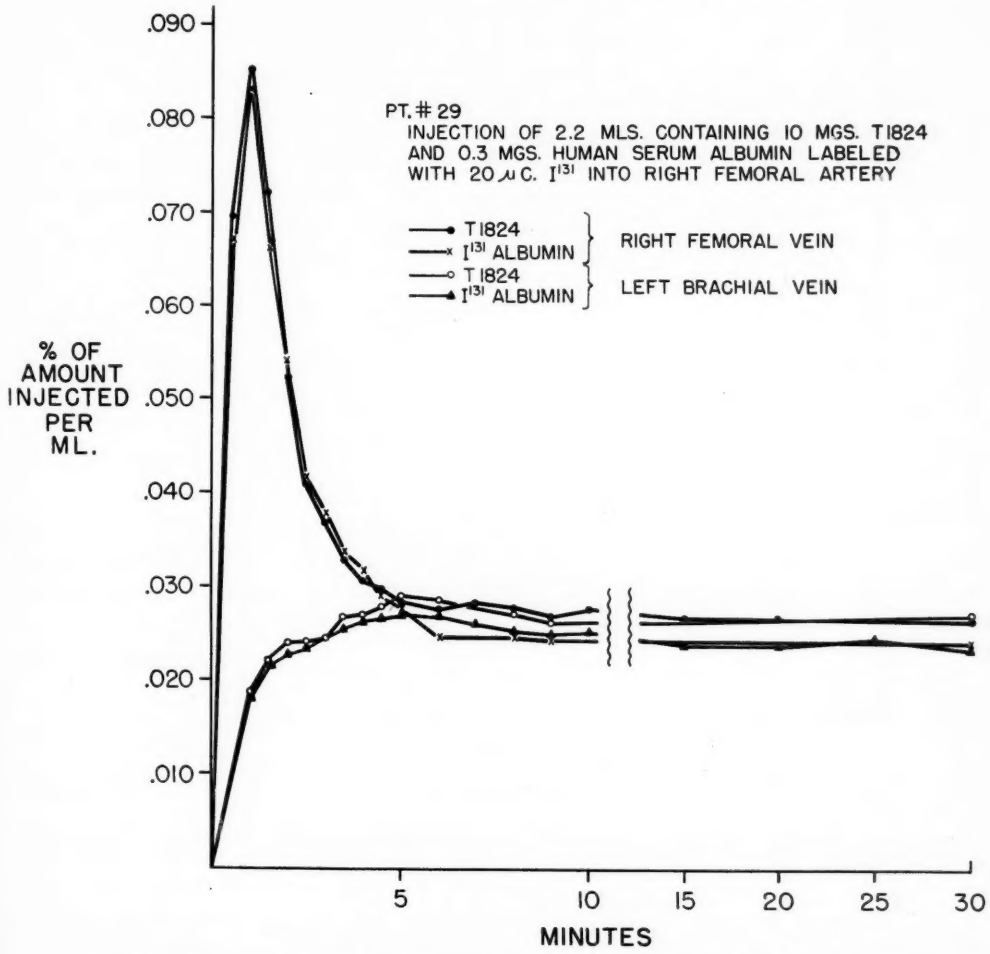

Fig. 1.—Distribution of dye and iodoalbumin following a single intra-arterial injection of a mixture of T-1824 and I^{131}-labeled human serum albumin. Manifest disparate removal of radioactivity occured after three minutes and was essentially complete within seven minutes following administration. Both indicators disappeared from the bloodstream at parallel rates during the subsequent thirty minutes.

iodinated albumin was mixed with T-1824 in molar ratios greatly exceeding these proportions. Attempts were made to define whether iodoalbumin required such excesses of dye for heterogeneous biologic behavior. Table II summarizes the physical properties of I.H.S.A., Lot No. 17, employed for this study.

In vitro characteristics were identical for undyed I.H.S.A. No. 17 or I.H.S.A. No. 17 combined 1:11 or 1:1,100 with T-1824. However, in the two

dogs given the 1:1,100 mixture, more than 10 per cent of the radioactivity was disparately lost within ten minutes, whereas the 1:11 or "dye-saturated" solution of I.H.S.A. behaved like undyed iodoalbumin in vivo (Table II).

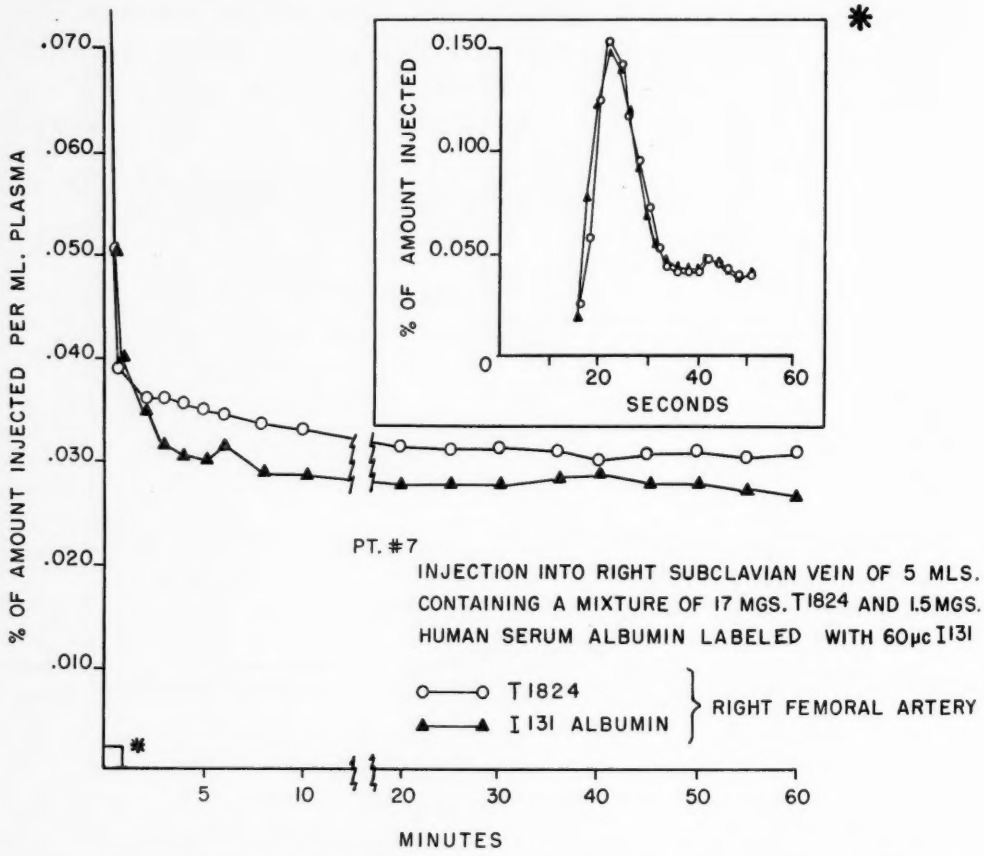

Fig. 2.—Distribution of dye and iodoalbumin following a single intravenous injection of a mixture of T-1824 and I^{131}-labeled human serum albumin. Disproportionate removal of radioactivity was observed after two minutes.

2. *In Vitro Studies.*—Dialyzable I^{131} exceeded 2 per cent of the total radioactivity in only two of seventeen separate lots of iodoalbumin; it was never greater than 3 per cent. Exchangeable iodide could be demonstrated occasionally when 5 per cent KI was substituted for distilled water in the dialysis bath. At such times, as much as 5 per cent of the total radioactivity could be recovered on the outside of the membrane. Addition of T-1824 in molecular ratios of dye:protein of 500:1 to 2,500:1 did not enhance the fraction of dialyzable or exchangeable I^{131}. Moreover, no progressive increase in dialyzable radioactivity could be demonstrated during storage of these sterile mixtures at 4° C. for as long as two weeks.

Fresh lots of iodoalbumin were tested for their content of butanol-extractable I^{131} by a technique which has been standardized in our laboratory to

recover 88-97 per cent of added thyroxine and to withhold all but 0.2 per cent of added inorganic iodide or diiodotyrosine.[13] Butanol-soluble I^{131} did not exceed the dialyzable radioactivity and only 0.2 to 0.4 per cent of the initial counts remained in the butanol phase following washing of acidified extracts with Blau's reagent. Addition of varying proportions of T-1824 to I.H.S.A. did not increase the butanol-extractable fraction.

TABLE II. PHYSICAL AND BIOLOGIC CHARACTERISTICS OF I^{131}-LABELED HUMAN SERUM ALBUMIN (LOT NO. 17). THE EFFECT OF PREMIXING WITH GRADED CONCENTRATIONS OF T-1824

I. Physical characteristics of albumin solutions	Cohn fraction V	Red Cross Albumin	I.H.S.A. (Lot No. 17)
A. Sedimentation constant ($S_w^{20} \times 10^{-13}$)	4.24	4.18	4.33
B. Electrophoretic mobility ($\mu \times 10^{-5}$) at pH 6.8 $\frac{r}{2}$0.1, 0° C.	4.4	5.1	5.0
C. Protein concentration (mg. %)	222	257	244
II. In vitro characteristics of mixtures:*	No. 1	No. 2	No. 3
A. Composition (molar ratio I.H.S.A. : T-1824)	1 : 0	1 : 11	1 : 1100
B. Dialyzable I^{131} (% of total radioactivity)			
1. 24 hour dialysis against distilled water	2.71	2.45	2.26
2. 24 hour dialysis against 5% KI	2.64	2.50	2.29
3. 120 hour dialysis against distilled water	2.74	2.73	2.04
4. 120 hour dialysis against 5% KI	2.85	2.39	2.38
C. Butanol soluble I^{131} after extraction with Blau's reagent (% of total radioactivity)	0.13	0.08	0.11
D. pH (units)	6.76	6.79	6.60

III. In vivo characteristics of mixtures: Simultaneous Distribution of I.H.S.A. and T-1824.† (Dog No.)	(9)	(10)	(11)	(12)	(13)	(14)
A. I.H.S.A. "space" (ml.)	682	416	568	620	631	673
B. T-1824 "space" (ml.)	691	425	574	609	548	590
C. Ratio T-1824/I.H.S.A.	1.013	1.022	1.011	0 982	0.868	0.877

*All mixtures were prepared with I.H.S.A. Lot No. 17.

†Additional T-1824 was administered into a contralateral vein in experiments with mixtures Nos. 1 and 2. T-1824 distribution was calculated from the dilution of the total amount of dye contained in the separate injections of T-1824 and the respective mixtures of I.H.S.A.

The pH of undyed and dyed solutions of iodoalbumin was measured with a Cambridge pH meter. Ph of the T-1824-I.H.S.A. mixtures ranged from 6.50-7.48.

All tested lots of I.H.S.A. moved as single components in electrophoresis and the ultracentrifuge. S_w^{20} for iodoalbumin ranged from 4.08 to 4.33 $\times$ 10.$^{-13}$ Simultaneous estimates of sedimentation constants for comparably diluted solution of Cohn Fraction V and Red Cross albumin varied from 4.08 – 4.24 $\times$ 10^{-13}. Optical difficulties precluded similar evaluations of mixtures of T-1824 and I.H.S.A. Light absorption was complete in both visible and ultraviolet spectra due to the presence of the large amounts of dye. Even removal of some of the unbound dye anions by extraction with butanol did not permit sufficient light transmission for adequate optical measurements. Paper strip methods were employed, therefore, to characterize the mixtures of dye and protein.

Undialyzed aliquots of dyed and undyed iodoalbumin were applied in 0.015 ml. amounts to 4 inch by 16 inch strips of Whatman No. 2 filter paper. Ascending chromatograms were prepared in a solvent system of butanol-acetic acid-water (200:30:75).[14] Strips were developed for sixteen hours, dried in a hood, and radioautographed on Eastman No-screen x-ray films. Most of the radioactivity remained at the origin. The mobile I^{131} migrated like inorganic iodide. Occasional batches of I.H.S.A. contained another small moiety which migrated with the solvent-front. Addition of massive amounts of T-1824 did not alter quantitatively or qualitatively the chromatographic-radioautographic behavior of the iodoalbumin in this solvent system. The studies were accepted as evidence against the liberation of tyrosyl residues from the albumin molecule by excessive quantities of T-1824.

Variously constituted mixtures of dye and labeled protein were subjected to microelectrophoresis according to the technique of Durrum.[15] Aliquots of 0.01 ml. were applied to the middle of 1 cm. wide dry strips of Whatman No. 2 filter paper which had been washed previously with distilled water. The strips were saturated with Veronal buffer at pH 8.6 and draped in a Lucite-enclosed case, between two Lucite vessels containing the buffer solution. A potential of 200 millivolts at eight milliamps was applied for 90 minutes to carbon electrodes inserted into the vessels. Each analysis was performed in duplicate. Strips prepared with solutions containing radioactivity were radioautographed upon Eastman No-screen x-ray film; all strips were subsequently sprayed with ninhydrin. Paper electrophoresis did not differentiate between native albumin and albumin labeled with I^{131}. Addition of massive amounts of T-1824 did not modify the microelectrophoretic characteristics of either type of albumin (Fig. 3). Unbound I^{131} could be demonstrated in all radioautographs of I.H.S.A. solutions. Most of the free radioactivity migrated as inorganic iodide. A small, discrete, unidentified fraction, however, could be localized immediately beyond the protein boundary. The presence of T-1824 did not enhance the magnitude of this unidentified fraction. It is of interest that in all radioautographs prepared with I.H.S.A., a shadowy zone could be observed extending from the point of application to the advancing margin of the albumin. This could not be clearly demonstrated with ninhydrin. Possibly the radioautographic image may represent an absorption of the carrier-poor I.H.S.A. to paper during migration which was quantitatively insufficient to yield a discernible ninhydrin reaction. Others have similarly observed such streaking during paper electrophoresis of I^{131}-labeled proteins.[16] Minor contaminants were occasionally demonstrated in the pure Oak Ridge solution of I^{131} which were used for control studies. Their precise nature remains obscure. Since all strips and utensils were scrupulously washed prior to usage, it seems unlikely that they represent iodination products produced during microelectrophoresis.

DISCUSSION

Despite some conflicting reports[17, 18] there are now many studies which have demonstrated that T-1824 and I^{131}-labeled albumin are equally distributed

during their earliest equilibration within the pool of exchangeable plasma proteins.[6, 11, 19] Similarly, in the present experiments, the rapidly exchanging albumin space could be measured with equal precision by the early dilution of T-1824 or I.H.S.A. when these indicators were administered by simultaneous but separate injection. However, a new phenomenon of disparate distribution has been described. Anomalous biologic behavior of iodoalbumin resulted from the prior mixing of labeled albumin with T-1824 in amounts calculated to

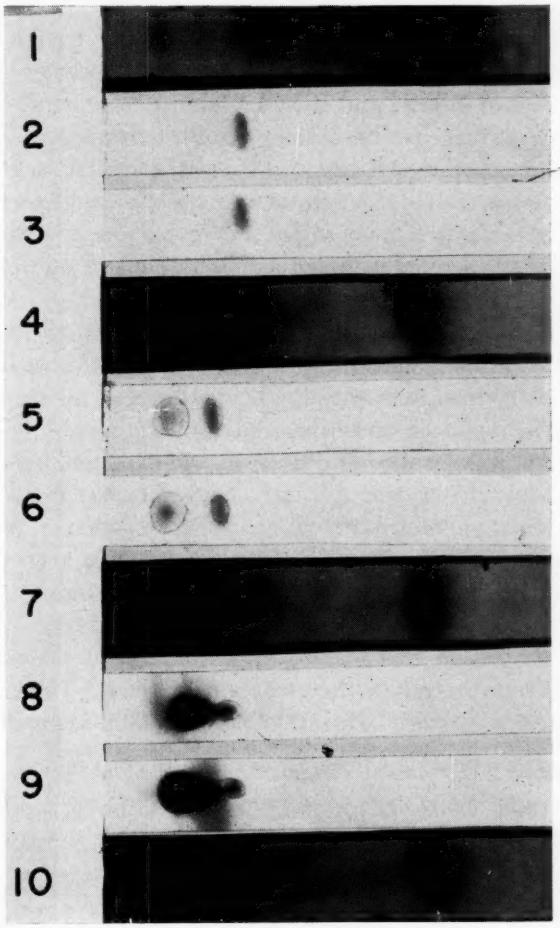

Fig. 3.—Paper electrophoresis of T-1824 and I^{131}-albumin mixtures. Electrophoresis was performed by the application of a potential of 200 millivolts at eight milliamps for ninety minutes to paper strips upon which had been placed 0.01 ml. aliquots of the following solutions: No. 1: Inorganic I^{131}. Radioautograph following ionophoresis.

Nos. 2, 5, 8: Purified Red Cross Human Serum Albumin, undyed (No. 2); combined 1:11 (No. 5); and 1:1,100 (No. 8) with T-1824. Strips were developed with ninhydrin. Site of application can be seen in No. 5 and No. 8 where T-1824 remained at origin.

Nos. 3, 6, 9: I^{131}-labeled human serum albumin (Abbott Lot No. 17), undyed (No. 3); combined 1:11 (No. 6); and 1:1,110 (No. 9) with T-1824.

Nos. 4, 7, 10: Radioautographs of Nos. 3, 6, and 9, respectively. Solutions of I.H.S.A. were not dialyzed nor enriched with carrier albumin prior to paper electrophoresis. Mixing with T-1824 did not enhance the radioactivity present with the inorganic iodide or with the unidentified moiety just distal to the albumin. In every instance, radioautographic streaking could be demonstrated between the point of application and the advancing margin of the protein.

bind no more than 5 per cent of the dye at pH 7.4. The manipulation uniformly enhanced the apparent dilution of the radioactive protein in vivo although both dye and radioactivity disappeared from the blood stream at parallel rates for the twenty minutes following intravascular equilibration. It was inferred that the apparently expanded I.H.S.A. space resulted from the early abstraction of an heterogeneous fraction of the iodoalbumin. The assumption was substantiated by demonstrating a disparate loss of I.H.S.A. from the blood stream during the first ten minutes following administration of I.H.S.A.-T-1824 mixtures. The site of removal could not be defined. Manifest operation of the process required at least three to four recirculations. Hence, the phenomenon did not invalidate studies of the first circulation based upon the concurrent dilution of mixtures of T-1824 and iodoalbumin.

The chemical nature of the moiety which is rapidly abstracted remains obscure. A macromolecular structure is indicated by the negative dialysis and chromatography experiments. Absence of disparate loss of radioactivity during the first circulation through the lower extremity also favors a macromolecular configuration since as much as 50 per cent of micromolecular solutes may leave the blood stream during such a transit.[20]

Allen and Orahovats have described an increase in the water content and a depression of the oxygen consumption of liver slices exposed to large amounts of T-1824.[21] Hemolysis of dog red blood cells has also been reported following exposure to T-1824 in concentration exceeding 1.3 mg./ml.[22]

In the present studies the addition of massive amounts of T-1824 introduced gross biologic heterogeneity into preparations of iodoalbumin. Any explanation for this phenomenon which is based upon a progressive photochemical or physiochemical alteration would not be in accord with the observation that neither in vitro properties nor in vivo disparities were altered during prolonged serial observations with the same mixture. The magnitude of the divergent distribution was seemingly a function of the individual mixture of dye and protein rather than the recipient subject. Thus, for example, the same combination of dye and I.H.S.A.—lot No. 3 administered at various times during an eight-day period to five different subjects yielded $\frac{\text{"T-1824 space"}}{\text{"I.H.S.A. space"}}$ ratios of 0.904, 0.898, 0.909, 0.875, and 0.923. Similarly, a single mixture prepared with I.H.S.A. lot No. 10 produced T-1824/I.H.S.A. ratios of 0.865, 0.882, and 0.841 in three subjects, whereas the same indicators were identically distributed when given uncombined.

Presumably, the close correlation with lot could be a function of the individual preparation of iodoalbumin per se or it could reflect the exact molar relationships with T-1824 which were established in the mixtures of dye and protein. The lack of precise measurements of the protein content of each lot of I.H.S.A. prevents separation of the two possibilities at present.

In each instance, early heterogeneity required an added amount of T-1824 far greater than those quantities conventionally accepted as necessary to bind all the available sites for dye-linkage in the albumin molecule. Conceivably, the huge excess of T-1824 may have exaggerated the formation of a

large molecular complex by displacing a subsidiary reaction of dye and protein to the right. Such a macromolecular aggregate would be rapidly abstracted from the circulation and would be formed in insignificant amounts at the low molar ratios of dye and protein which are normally established in the blood stream. If such complex formation occurs, repetition of these studies with other preparations of labeled albumin and more rigid control of pH at 7.4 may reveal whether it is peculiar to Abbott iodinated proteins or whether it is of general importance in the interaction of T-1824 with albumin.

SUMMARY

Addition of massive amounts of T-1824 to electrophoretically and ultracentrifugally homogeneous preparations of I^{131} human serum albumin (Abbott) did not change characteristics of the labeled protein in vitro as judged by serial dialyses, butanol extractions, paper chromatography and paper electrophoresis. This manipulation, however, significantly altered the biologic behavior of iodoalbumin.

Distribution of I.H.S.A. exceeded that of T-1824 during the first thirty minutes following the administration to man and dog of mixtures in which T-1824 and iodoalbumin had been combined in molar proportions exceeding 500 parts of dye to one part of protein. The expanded "I.H.S.A. space" resulted from a disparate removal from the circulation of some of the radioactivity during the first ten minutes. Absolute losses ranged from 4 to 28 per cent of the total counts and were related to the individual mixture rather than the experimental subject. Early metabolism of the abstracted moiety could not be demonstrated by increased urinary excretion of I^{131} within the first twenty-four hours. When the same preparations of I^{131}-labeled albumin and T-1824 were given by separate administration or in mixtures of 11 parts of dye to 1 part of protein, the ratios of $\frac{\text{"T-1824 space"}}{\text{"I.H.S.A. space"}}$ approximated unity.

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THE CYTOLOGY OF THE DEGENERATING LEUKOCYTE

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IN A RECENT report of a new stain for urinary sediments, Sternheimer and Malbin[28] described an alcoholic mixture of gentian violet and safranin O, which permits easy recognition of the cellular and formed elements of urinary sediment. Certain leukocytes in urinary sediments stained with this mixture are described as relatively large, pale blue-staining cells characterized by their tendency to variability in size and shape, to vacuolization, extrusion of fragments of cytoplasm, and to Brownian movement of their cytoplasmic granules. There was reported a correlation between the occurrence of these cells in the urine and the presence of pyelonephritis, as established by clinical observation and post-mortem findings in twenty-five cases.

Such leukocytes have been observed by many workers. Schilling[24] described the "glittering leukocytes" of the urine, Sabin[21, 22] and others[10, 27] pointed out similar cells in supravital preparations of blood, and Crosbie[2] observed such cells in citrated stored blood. The described changes are limited to the polymorphonuclear neutrophilic leukocytes and are not exhibited by eosinophils, basophils, or any cells of the mononuclear series. Most observers have considered such leukocytes to be dead or dying.

It is the purpose of this investigation to determine the mechanism responsible for the appearance of such leukocytes, correlating morphologic changes with cytochemical processes.

METHODS AND RESULTS

A. Direct Staining Methods.—The direct staining of leukocytes was first carried out using the stain proposed by Sternheimer and Malbin. The stock solutions were prepared as follows:

Solution I:	Gentian Violet (85 per cent dye content)	
	Crystal Violet	3.0 Gm.
	95 per cent Ethyl alcohol	20.0 ml.
	Ammonium oxalate	0.8 Gm.
	Triple distilled water	80.0 ml.
Solution II:	Safranin O (95 per cent dye content)	
	Safranin O	0.25 Gm.
	Alcohol 95 per cent	10.0 ml.
	Triple distilled water	100.0 ml.

Ninety-seven parts of the safranin stock solution were mixed with three parts of the gentian violet stock solution and the mixture was filtered. The resulting mixture thus contained approximately 0.1 per cent crystal violet and 0.2 per cent safranin O in alcoholic solution. Staining was carried out by mixing a small drop of centrifuged urinary sediment or other material with a small drop of stain. A drop of this mixture was then placed on a slide, covered with a cover slip and examined under oil immersion.

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Urinary sediments stained by this method were found to contain leukocytes which stained deeply, containing dark red to purple nuclei and violet to purple cytoplasm, the granules of which showed no Brownian movement. In known pyelonephritic urine, in addition to these deeply stained leukocytes, other leukocytes were observed to be larger with spherical or multilobulated pale blue nuclei and unstained cytoplasm containing slate gray granules which exhibited marked Brownian movement. These leukocytes were observed to be polymorphonuclear neutrophils. Hereinafter, we will refer to this latter type of cell as a granular motility cell or G.M.-cell.

In an attempt to study larger numbers of leukocytes stained by this method, various preparations were observed employing blood from normal donors. A small drop of fresh finger-tip blood to which was added a drop of the stain mixture was not found to contain granular motility cells, at least not in detectable numbers. The buffy coat pipetted from centrifuged blood preserved in mixed oxalate, however, was found to contain large numbers of granular motility cells. Freshly heparinized blood buffy coat likewise contained G.M.-cells, though apparently in less proportion. Freshly heparinized and freshly oxalated blood buffy coat suspended in normal saline approximating the original volume of blood and recentrifuged contained even greater numbers of G.M.-cells.

The effect of tonicity in producing G.M.-cells was studied by observing a small drop of finger-tip blood after incubation in varying concentrations of saline at 37° C. for thirty minutes. The results are shown in Table I. As the concentration of saline became more hypotonic or hypertonic, more G.M.-cells were observed, provided the osmolarity of the solution remained within a range such that the leukocytes were not fragmented.[30] On the basis of these observations it would appear that the G.M.-cell is a polymorphonuclear neutrophilic leukocyte, which has undergone nonspecific damage.

TABLE I. EFFECT OF TONICITY IN PRODUCING G.M.-CELLS

NaCl CONCENTRATION (PER CENT)	APPEARANCE OF LEUKOCYTES
1.80	G.M.-cells observed in small numbers
1.44	No G.M.-cells
1.08	No G.M.-cells
0.90	No G.M.-cells
0.72	Many G.M.-cells
0.36	Predominantly G.M.-cells

Suspension of a small drop of finger-tip blood in 5 ml. of 0.72 per cent saline at room temperature was found to produce a sufficient quantity of G.M.-cells for observation without destroying large numbers of cells. Blood suspended in this medium was centrifuged, the supernatant decanted and the cells observed in various stain solutions. Gentian violet solution was prepared by adding 3 parts of gentian stock solution to 97 parts of the safranin diluent not containing the safranin O so as to produce a stain identical to the gentian-safranin mixture in concentration of all constituents with the omission of

safranin. Granular motility cells stained with this solution were indistinguishable from those stained with the original gentian-safranin mixture, exhibiting pale blue nuclei, unstained cytoplasm and gray, unstained granules with marked Brownian movement. Other cells were noted to contain deep blue nuclei, light blue cytoplasm, and no active granules. The safranin O stock solution was combined with the gentian diluent to produce a concentration of safranin as in the gentian-safranin mixture but with the gentian omitted. With this stain, G.M.-cells were unstained; other cells were observed to contain orange-red nuclei and yellow-pink cytoplasm with nonmotile granules. Thus both dyes in the mixture participate in the staining of the darker cells, producing the typical purple color. The gentian violet acts as a stronger dye, however, and it alone stains the nuclei of the G.M.-cells, leaving the cytoplasm unstained by either dye.

Both gentian violet and safranin O are basic dyes. The process of staining with such basic dyes depends upon chemical combination with acid cellular constituents.[16] The failure of the G.M.-cell to stain as deeply as other cells indicates a loss of the acid cellular constituents responsible for such staining. Since the nucleus does stain slightly, the dye must have entered the cell, hence impermeability of the cell membrane to the dye cannot be considered to be the primary mechanism in the failure of the cell to stain.

Granular motility cells fail to stain in a solution of 1 per cent trypan blue. The dense, diffuse staining of a cell nucleus by trypan blue is a fairly well-accepted criterion of fatal injury of a cell.[23] Hence G.M.-cell can be considered damaged, but not fatally injured cells.

A solution of 0.1 per cent fast green, an acid dye, was found to stain the cytoplasm of the G.M.-cells a very pale yellow-green. The cytoplasm of other cells with nonmotile granules also stained a pale green. The nuclei remained unstained in all cells. There was no differentiation of cytoplasmic structures by any of these direct staining techniques, the cytoplasm either staining diffusely, or not staining at all.

B. Supravital Staining.—Observations of supravital preparations of fresh finger-tip blood were carried out according to the method of Schwind.[25] A small drop of blood was placed on a clean cover slip and dropped on a slide previously prepared by having dried on its surface a solution of neutral red and pinacyanole in absolute alcohol. The weight of the cover slip pressed the blood layer to about one-cell thickness and the edges of the cover slip were then sealed with petrolatum.

The nuclei of the polymorphonuclear neutrophils stained a pale blue with pinacyanole[19] and the neutrophilic granules stained a pale pink with neutral red. Most neutrophils were seen to be actively ameboid, and the neutral red-staining granules could be seen streaming into the projecting pseudopodia. Other neutrophils were stained similarly but were rounded up, not actively ameboid, being in the so-called "resting state." Still another type of neutrophil was the rounded form in which the cytoplasmic granules were unstained and exhibited marked Brownian motion. This cell is identical in appearance with the previously described granular motility cells. Furthermore, if actively

ameboid, well-stained neutrophils or those in the "resting state" were observed over a period of several hours, the following changes occurred. Small vacuoles deeply stained with neutral red began to appear in the cytoplasm and became progressively larger. They were one to several in number and were stained a deep orange-red. As these vacuoles became progressively larger, the cell began to round up, showing less ameboid activity. The neutrophilic cytoplasmic granules were seen to become progressively lighter in color until the smaller granules appeared unstained and began to show Brownian movement. Thus the actively ameboid leukocyte over a period of hours could be seen to become identical to the G.M.-cell with the exception that the cytoplasmic basophilic material had been gathered into large vacuoles or globules deeply stained with neutral red.

The formation of similar vacuoles by the action of basic dyes or ammonium compounds on mammalian reticulocytes has been described by Dustin.[5] He found that after fixation and removal of the vital dye, a network appeared where the vitally stained vacuoles had been in the living cell. This network stained intensely with all basic dyes. The histochemical study of this substance indicated that it contained ribonucleic acid.

The usual supravital preparation of blood was not found to lend itself well to the manipulation of counterstaining due to the fact that most of the leukocytes were destroyed in the process of removing the cover slip. The older more damaged cells containing larger neutral red vacuoles were especially prone to show fragmentation. A modified supravital staining technique was then employed to obviate the use of a cover slip. A small drop of blood was placed on a slide or cover slip bearing the precipitated stain mixture of neutral red and pinacyanole or neutral red alone. The drop of blood was then smeared over a small area with the corner of a slide, much as in making a thick smear for malaria. The smear was then quickly inverted over a concavity slide and the edges of the two slides sealed with Scotch Tape to prevent drying.

After allowing sufficient time for the neutrophils to form neutral red vacuoles (one to five hours at room temperature) the slides were separated. The smears were then allowed to dry in air or were fixed wet in either 95 per cent methyl alcohol or Carnoy's fluid. The neutral red was removed by rinsing two minutes in 95 per cent methyl alcohol. The smear was then stained with 1 per cent toluidine blue O in 95 per cent alcohol, rinsed in distilled water, and allowed to dry. In addition to deep blue nuclei, small blue-stained granules were seen in the cytoplasm in positions formerly occupied by the neutral red vacuoles. This would indicate that ribonucleic acid is a constituent of the neutral red vacuoles in accordance with the work of Dustin. Toluidine blue O in particular was chosen to restain the preparations after the removal of neutral red, because toluidine blue O, in addition to its staining action as a basic dye, shows the property of metachromatic staining.[16] It stains nucleic acid blue, or orthochromatically, whereas it stains mucin purple to lavender, or metachromatically, thereby providing additional differentiation of cytoplasmic acid constituents.

C. Histochemical Methods.—Smears prepared by the above technique were then examined under oil immersion. Leukocytes containing suitable granules were located on the slide for further observation by recording their positions with a reference slide bearing numbered coordinates or encircling them with a diamond-tipped stylus. The oil was removed by rinsing in xylene and 95 per cent alcohol. The smear was then decolorized by rinsing in acetone, water, 50 per cent alcohol, and water, in that order.

The ribonucleic acid was removed by a modified ribonuclease process described by Kurnick.[12] This consisted of immersion of the slide for twenty-four hours in 0.05 M. trimethylol aminomethane buffer at 21° C. The smear was restained with toluidine blue O and the previously observed cells were re-examined. The nuclei were again stained a deep blue indicating that the desoxyribonucleic acids were still present. However, the cytoplasmic granules which were observed at the positions formerly occupied by the neutral red vacuoles had disappeared. This indicated that these granules were composed of ribonucleic acid, having been removed by the action of the buffer.

DISCUSSION

Certain polymorphonuclear neutrophilic leukocytes in unfixed preparations have been observed to be larger, containing rounded or multilobular nuclei, and cytoplasmic granules exhibiting marked Brownian motion. Such cells have been described in both urine and blood and have been variously referred to as glittering leukocytes,[22] nonmotile leukocytes,[2] rounded forms,[10] spinning leukocytes,[2] or granular motility cells.[28] Attempts have been made to correlate the appearance of such cells with a normal rhythm of the leukocytes of human blood.[9, 22, 27] "Showers" of such nonmotile leukocytes have been experimentally produced by the intravenous injection of sodium citrate into normal rabbits.[1] The presence of such cells in the urine has been correlated with the presence of pyelonephritis.[24, 28]

Brownian movement of cytoplasmic granules has been described after injury of other types of cells[15] also and seems to be rather well correlated with the apparent fluidity of the cytoplasm.[18] In studies on the relative effects of different concentrations of anisotonic media, the phenomenon of increasing apparent fluidity (with resulting increase in Brownian movement of cytoplasmic particles) has been shown to depend on the shearing stress and is probably due to the structure causing elasticity.[18] Thus damage to cellular structure by any method which reduces the elasticity of the cytoplasm or causes a decrease in viscosity should cause an increase in Brownian activity of cytoplasmic particles. Therefore Brownian movement of the cytoplasmic granules of neutrophilic leukocytes seems to be rather well correlated with nonspecific damage from both experimental and theoretical viewpoints.

As neutrophils become progressively more damaged, the cytoplasmic granules are seen to lose their affinity for basic dyes. This phenomenon was first thought to occur because the cell membrane had become impervious to the dye.[21] However, later observations indicated that the granules have actually lost their power to react to the dye. This is evidenced by the fact that upon rupture of the cell membrane, the granules are freed into the surround-

ing fluid, yet remain entirely unstained.[22] This latter observation is borne out by both wet staining and supravital staining techniques. In wet direct staining techniques, the cytoplasm of damaged cells is seen to remain unstained, whereas undamaged cells readily accept the stain. In supravital techniques, as stained cells become damaged they are seen to develop large deeply staining vacuoles. Meanwhile, the granules which were formerly stained become progressively less stained, some becoming entirely unstained. This vacuolization power of cells has been referred to as the segregating action of the cell.[6] Cells damaged prior to staining show no affinity for the supravital dye in any portion of the cytoplasm, neither granules nor vacuoles. This indicates that the basophilic substance has been either removed or sufficiently altered so that it no longer reacts with the basic dye. The cytoplasmic substance responsible for this basophilia of neutrophilic granules has been shown to be ribonucleic acid. This was demonstrated by histochemical analysis of the basophilic material segregated into cytoplasmic vacuoles by the action of neutral red.

There is a striking similarity between the cystoplasmic granules formed by the fixation of neutral red vacuoles restained with toluidine blue O, and the toxic granules seen in neutrophils during bacteremic states.[11, 20, 29] Thin blood films from a patient with bacterial endocarditis were examined and found to contain large numbers of neutrophils containing typical toxic granules. These toxic granules stained darkly with toluidine blue O and were removed by the modified ribonuclease method described above. In this particular case, the toxic granules are shown to be composed of ribonucleic acid, at least in part, if not entirely.

Toxic granulation has been variously ascribed to histologic degenerative change, the toxic effect associated with suppuration, and phagocytosed particles. From this histochemical study of the degenerating leukocyte, it is conceivable that such toxic granulation may occur by the segregation of ribonucleic acid much as in the supravital staining with neutral red.

Although toxic granulation is evidently produced by bacteremia, the exact relation of bacteria and bacterial products to the segregation of cytoplasmic ribonucleic acid into toxic granules has yet to be shown.

CONCLUSIONS AND SUMMARY

Certain polymorphonuclear neutrophilic leukocytes found in urine have been correlated with the presence of pyelonephritis. These cells are characterized by their larger size, poor staining qualities, tendency to variability in size and shape, vacuolization, extrusion of cytoplasmic fragments and Brownian movement of their cytoplasmic granules. Such cells have also been observed in normal blood in small numbers.

By observing the effects of various methods of blood preservation and the effects of tonicity, the appearance of these leukocytes has been shown to result from nonspecific damage. Such damage decreases the cytoplasmic viscosity producing Brownian movement of the cytoplasmic granules.

Supravital studies show that concomitantly with the appearance of Brownian movement of the cytoplasmic granules, the granules progressively lose their affinity for neutral red. The neutral red can then be seen to collect in deep staining vacuoles which become larger and more deeply stained as the cytoplasmic granules lose their stain. Histochemical study has shown that these deeply stained vacuoles contain ribonucleic acid.

Damage to neutrophilic leukocytes has been shown to be intimately associated with the loss of ribonucleic acid from the neutrophilic granules. This results in the loss of basophilia of the granules.

Neutral red vacuoles, when fixed and the neutral red removed, exhibit granules which stain deeply with basic dyes. There has been shown a striking similarity between these granules and the toxic granules observed in the neutrophils during various bacteremic states. It has been suggested that such toxic granulation may occur by the segregation of ribonucleic acid much as in the supravital staining with neutral red.

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CHARACTERISTIC CELLULAR CHANGES IN EPITHELIAL CELLS IN PERNICIOUS ANEMIA

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THIS paper is a report of specific cellular changes occurring in the epithelial cells of patients with pernicious anemia.

The morphology of the red blood cell in pernicious anemia has received a great deal of attention and the alterations are well known. The white blood cells have distinct morphologic changes, but very little attention has been paid to any other type of cell than those of the peripheral blood. Epstein[1] in 1949 reported marked cellular changes in the megakaryocytes of the bone marrow in patients with this disease. The predominant changes were a marked increase in size of the cells and multinucleation. These changes were reversible, disappearing after the institution of liver therapy.

Although there has been clinical evidence that the squamous epithelium of the oral cavity must be undergoing certain changes, since glossitis is so often present, no specific study of the cellular changes has been done, perhaps because biopsy seems an unnecessarily rigorous investigative method. In the last ten years, however, the morphology of the desquamated cell from all types of epithelium has been intensively studied. With the advent of the cytologic method for the diagnosis of cancer, and its application to many organs, a method simple in technique became available to study single cell morphology.

The Vincent Memorial Laboratory examines large numbers of smear preparations from the vagina, lung, stomach, and bladder. During the routine examination of sputum and gastric juice for the presence of cancer cells, our attention was rather forcibly drawn to the fact that patients with pernicious anemia had specific cellular changes in squamous epithelial cells. In fact, the first time these markedly atypical cells were seen, the sputum smear was mistakenly called "positive, consistent with a squamous carcinoma." Since these cellular changes almost completely disappeared during therapy after the hemoglobin rose to a satisfactory level, the smears of other patients with pernicious anemia were re-examined. This paper is a report of that investigation.

The material for this study came from routine gastric cytologic examinations. In the last six years the sediment of the gastric secretion of 1,600 patients has been studied microscopically. In these 1,600 cases there were 41 cases of proved pernicious anemia.* Of these 41 cases, 20 were in relapse. Fifteen of these were being studied for the first time. The remaining 21 were in therapeutic remission. All patients had severe macrocytic anemia before treatment. With one exception, all patients in relapse had hemoglobin values below 8.5 Gm. per cent. Of the 21 cases in remission, all had hemoglobin

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values above 12.5 Gm. per cent. All showed a therapeutic response to liver or B_{12} therapy. No patient had free hydrochloric acid in the gastric secretion after histamine.

As control cases, four cases of severe iron deficiency anemia and three cases of acute blood loss were studied. In three of the cases of severe anemia of iron deficiency origin, the hemoglobin values were below 7.5 Gm. per cent. The fourth case had a value of 9.5 Gm. per cent. In the three cases with acute blood loss, the values were 9.2, 8.5, and 6.3 Gm. per cent. Free hydrochloric acid was found in the gastric secretion of three patients, two of whom had iron deficiency, and one with acute blood loss.

METHOD

The method we use in obtaining gastric secretion for cytologic study is as follows: A No. 14 Levine tube with multiple holes is introduced into the stomach through the nose. The patient may drink water to facilitate the passage of the tube. The patient must have taken nothing by mouth after 7:00 P.M. the previous evening. All secretion in the fasting stomach is aspirated. This is specimen No. 1. One hundred milliliters of saline are introduced into the stomach through the tube. The solution is allowed to remain fifteen minutes and during this time the patient is asked to turn from side to side several times. At the end of fifteen minutes, all solution is withdrawn. A great effort is made to reclaim a volume of fluid equal to that introduced. This is specimen No. 2.

The two specimens are taken to the laboratory immediately. It is essential that no time be lost in transportation since the cells deteriorate rapidly. In the laboratory the two specimens are centrifuged in separate tubes and the sediment from each is placed on two marked slides. Thus, from each patient there are four slides. They are placed in a fixative of equal parts alcohol and ether. They must remain in the fixative for at least a half hour. They are stained by the method of Papanicolaou.[3]

RESULTS

It should be remembered that the majority of cells seen in the routine gastric aspiration do not come from the stomach itself. The gastric juice contains predominately cells which have been swallowed. These cells desquamate from the upper respiratory and gastrointestinal epithelium and are squamous in origin. They are superficial squamous cells. In cytologic terminology, a superficial squamous cell is a large flat cell with a large amount of cytoplasm surrounding a rather small single nucleus which is central in position. The nucleus may be finely vesicular in appearance or completely pyknotic. (Fig. 1.) These cells form the top, or superficial layer of squamous epithelium.

These superficial cells show marked changes in patients with untreated pernicious anemia. The most prominent change is an increase in size of both nucleus and cytoplasm. The superficial squamous cells in pernicious anemia average 16 microns larger in their greatest diameter than those of normal patients or in the patient with pernicious anemia in remission. (Fig. 2.)

Another marked change occurring in these cells is multinucleation. This occurs in the cells which are increased in size. Fig. 3 shows this type of change. It can be seen that this cell has had a severe interference with its normal mitotic division. There are five nuclei contained within this one cytoplasmic unit.

The third change is a change in the chromatin distribution of the cell. The normal nuclear configuration is an even one. In some of the squamous cells in untreated pernicious anemia, the chromatin becomes very active-looking and is in large clumps. In Fig. 3 it can be seen that neither the chromatin content nor the nuclear pattern is the same in all five nuclei. Fig. 4 shows three enlarged cells with increased chromatin arranged in uneven large clumps.

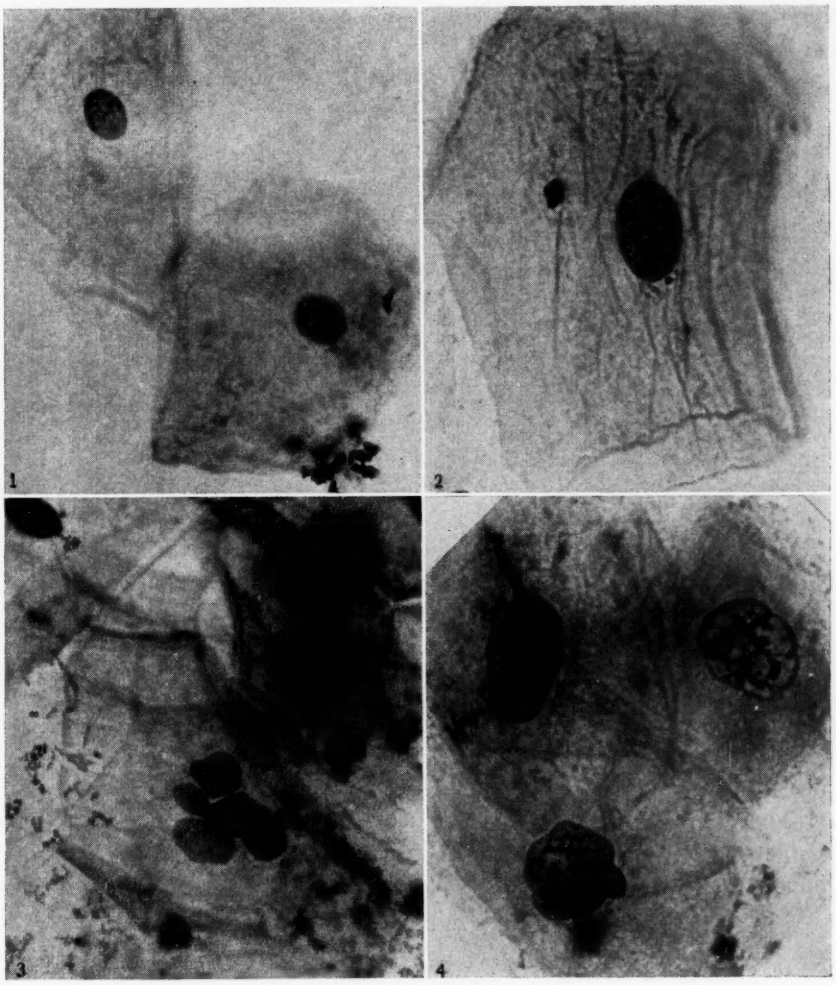


Fig. 1.—Normal superficial squamous cells.
Fig. 2.—Superficial squamous cell exhibiting marked size increase in both nucleus and cytoplasm. Compare with cells in Fig. 1.
Fig. 3.—Large epithelial cell containing five nuclei in one cytoplasmic unit.
Fig. 4.—Three epithelial cells with large nuclei showing abnormal chromatin arrangement.

The cell of most interest in gastric secretion is the columnar cell. It is an elongated cell with an eccentric vesicular nucleus. Usually in gastric secretion they have a poorly preserved cellular border. The cytoplasm is irregular or vacuolated in appearance. Their eccentric nuclei and elongated form iden-

tify them as coming from the gastric epithelium. They can be distinguished from the columnar cells coming from bronchial epithelium by their lack of cilia. (Fig. 5.)

These cells when seen in the gastric secretion of untreated pernicious anemia patients also exhibit the same specific changes as seen in the squamous epithelial cell. They increase in size, both in length and breadth. (Figs. 6 and 7.) They have multiple nuclei as shown in Fig. 7. The chromatin material of these nuclei is markedly increased and it is abnormally distributed.

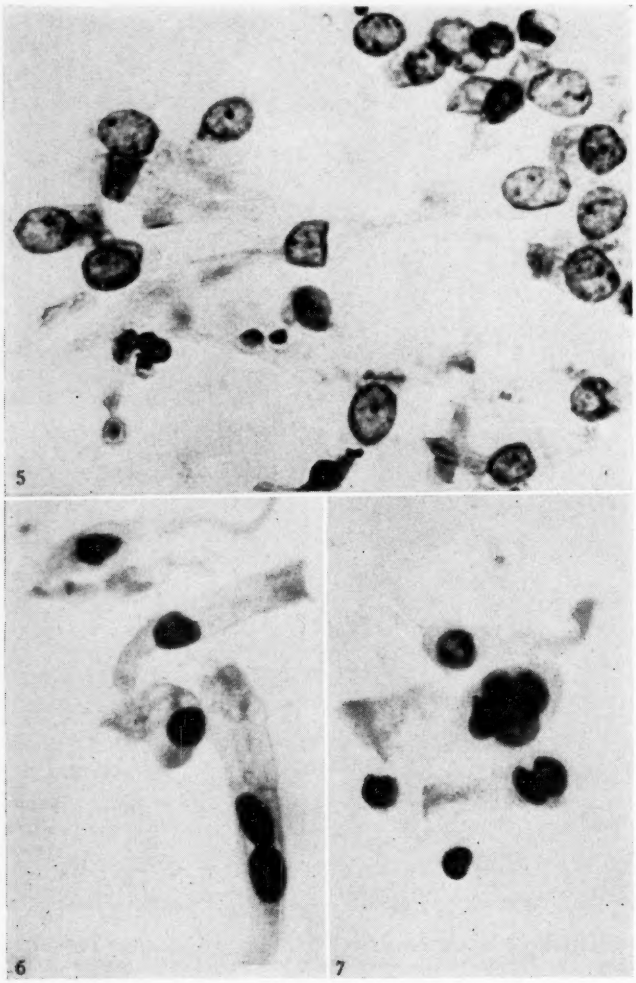


Fig. 5.—Group of normal columnar cells with even nuclear configuration and wisps of degenerating cytoplasm.

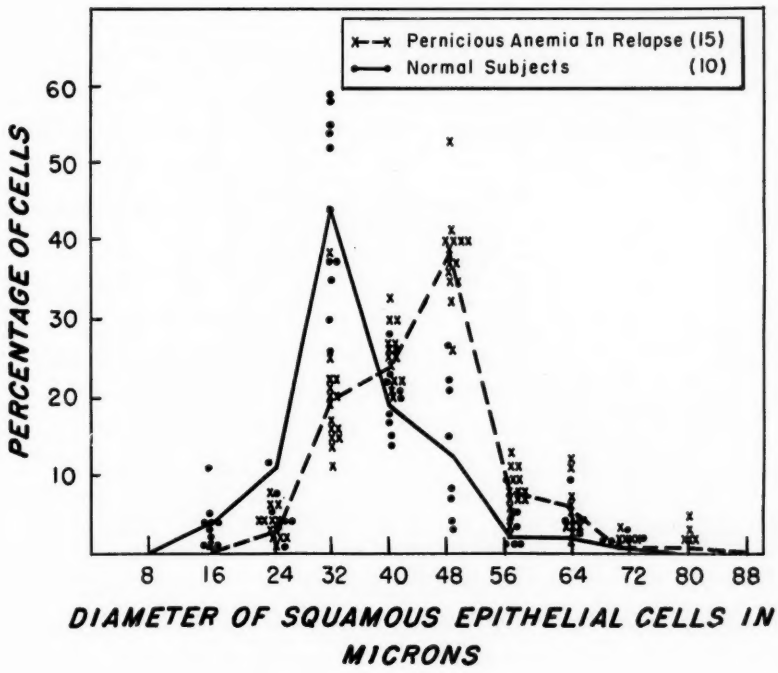
Fig. 6.—Four elongated atypical columnar cells seen in untreated pernicious anemia. Compare with Fig. 5.

Fig. 7.—Columnar cell in center of field shows increased width and multiple nuclei.

Compare Figs. 6 and 7 with Fig. 5. This increase in chromatin and change in nuclear configuration is especially interesting, in view of the known increased incidence of gastric carcinoma in the pernicious anemia group.

It can readily be seen from the photomicrographs that these are definite cellular changes. It was of real interest to us that after either liver or vitamin B_{12} medication was given, these changes markedly decreased. They were almost completely absent in the patient in clinical remission. It appeared difficult to judge such changes or lack of changes quantitatively. This problem was discussed with Miss Geneva Daland and Dr. William Castle of the Thorndike Memorial Laboratory, and, at their suggestion, Price-Jones curves were done to determine if there was an increase in size in all cells.

CHART I

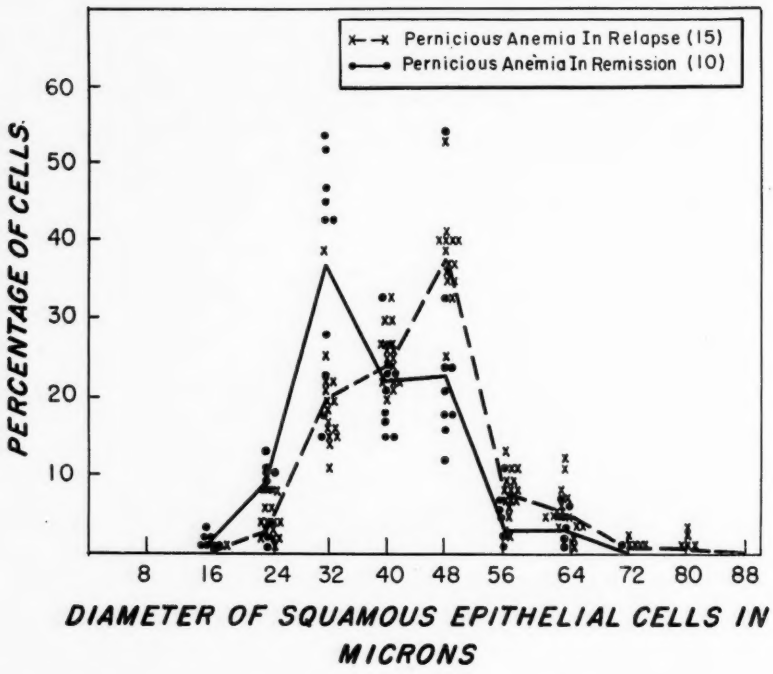


One hundred consecutive superficial squamous cells were measured in their greatest diameter and the number of cells of different measurements plotted against the diameter in microns. One hundred cells were measured in fifteen patients with proved pernicious anemia before any treatment. As controls, one hundred cells were measured in ten normal patients. The controls were chosen as patients with adequate hemoglobin values, negative gastrointestinal series, and free hydrochloric acid in the gastric secretion. Chart I compares the results in cell measurements in the two groups. The peak of the curve for normal cells is at 32 microns and the range from 16 to 64 microns. In contrast, the squamous epithelial cells in untreated pernicious anemia are much larger, the peak being at 48 microns and the range from 24 to 80 microns.

It seemed of interest to determine if the size of the squamous cells in patients of pernicious anemia in remission reverted to normal. Therefore the

squamous epithelial cells of a third group of ten patients in therapeutic remission were measured. Chart II shows this group in comparison to the untreated group. It can be seen by comparison of Charts I and II that the cells have decreased in size in the treated group and very nearly approach the normal.

CHART II



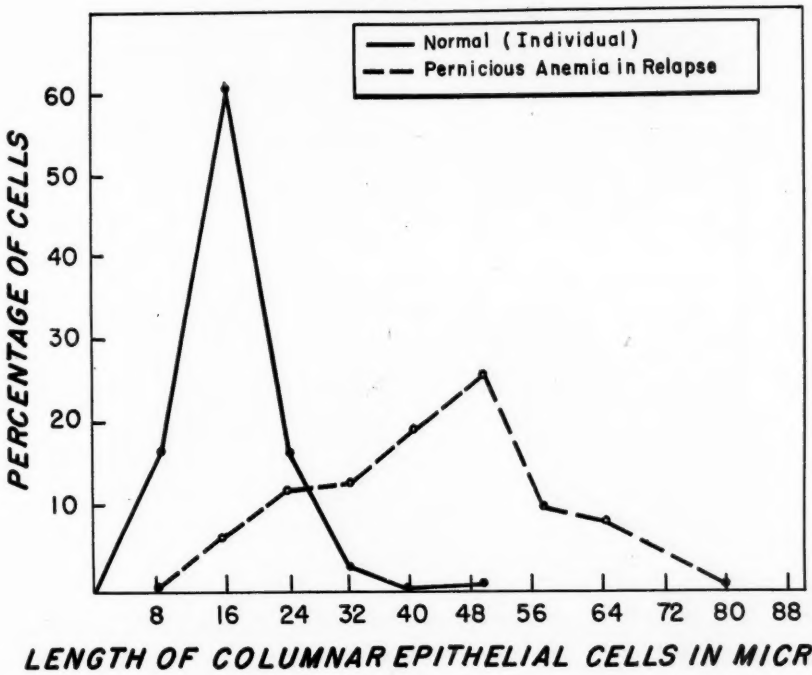
As mentioned above, the columnar cells from the gastric mucosa are also atypical when seen in the gastric smear. Unfortunately these cells do not occur in great numbers in the routine aspiration and it was impossible to find enough cells to measure except in one case of untreated pernicious anemia. One hundred consecutive columnar cells were measured in their longest diameter, and compared to the same type of measurements on columnar cells in a normal patient. Chart III shows this comparison. It is obvious that the size of normal columnar epithelial cells does not vary as in the case of normal squamous epithelial cells. The reason for this is that normal squamous epithelial cells increase in size as they get nearer the surface of the epithelium. Columnar cells, on the other hand, come from an epithelium which is only a single cell layer thick and there is much less chance for variation in size.

There is a marked difference in the length of normal columnar cells and in those encountered in pernicious anemia. The peak of the curve for normal columnar cells is 16 microns, in contrast to 48 microns in the pernicious anemia patient. The spread is very little in the normal, from 8 to 32 microns, while

in pernicious anemia it is from 16 to 80 microns. Though this observation is only on one case, it would appear to indicate that the size of columnar cells is affected in the same way as squamous cells in untreated pernicious anemia.

Occasionally, in the gastric smear, phagocytic histiocytes from sputum are encountered. These are histiocytes containing carbon particles, the so-called "dust" cells of sputum. These occur in the gastric secretion when sputum is swallowed. (Fig. 8.) In one of the cases of untreated pernicious anemia patients in this series, such cells were seen. This is fairly uncommon since a patient with untreated anemia must be producing sputum and swallowing it.

CHART III



Consequently we have only one such case where this type of cell could be measured. This was done, and Chart IV compares the size of these phagocytic histiocytes in untreated pernicious anemia (Fig. 9) with the same cell type in the gastric secretion of a normal individual (Fig. 8). Again, there is a marked difference. There is little variation in the size of these cells in the normal patient, 82 per cent of them measuring 16 microns. However, in the untreated anemia patient, the peak of the curve is at 40 microns, indicating at least a twofold increase in cell size.

It can be concluded from the four charts shown that the squamous epithelial cells, the columnar epithelial cells, and the histiocytes in untreated pernicious anemia are markedly increased in size. Also, at least in the instance of the squamous epithelial cells, the size reverts to normal after liver or vitamin B_{12} therapy.

But what of the other cellular changes? Size increase is only one of the changes seen in the squamous epithelial cells. The multiple nuclei and change in nuclear pattern are important alterations. In an attempt to determine semi-quantitatively if these changes reverted to normal, 500 consecutive squamous epithelial cells were counted in 18 cases of untreated anemia, and 18 cases in

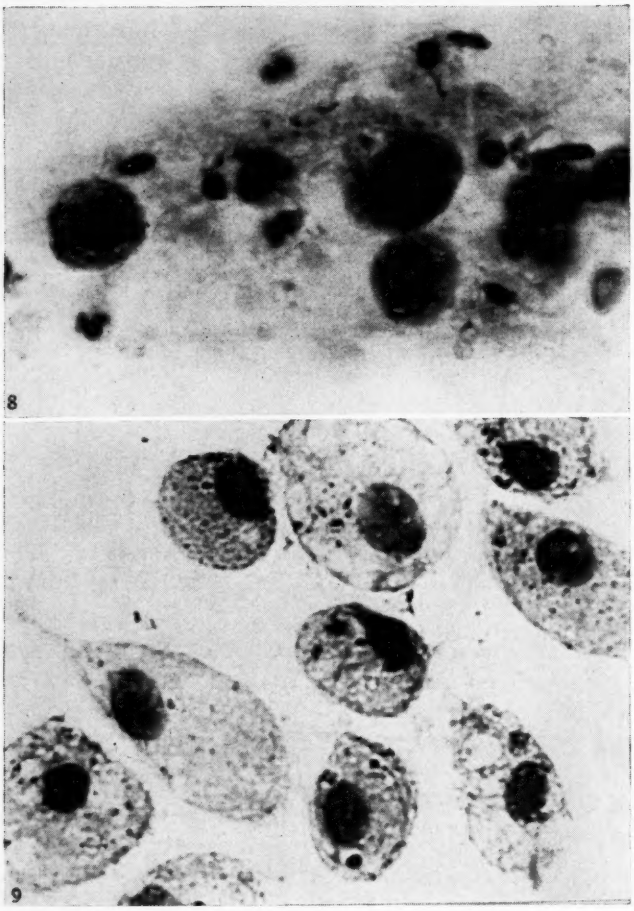
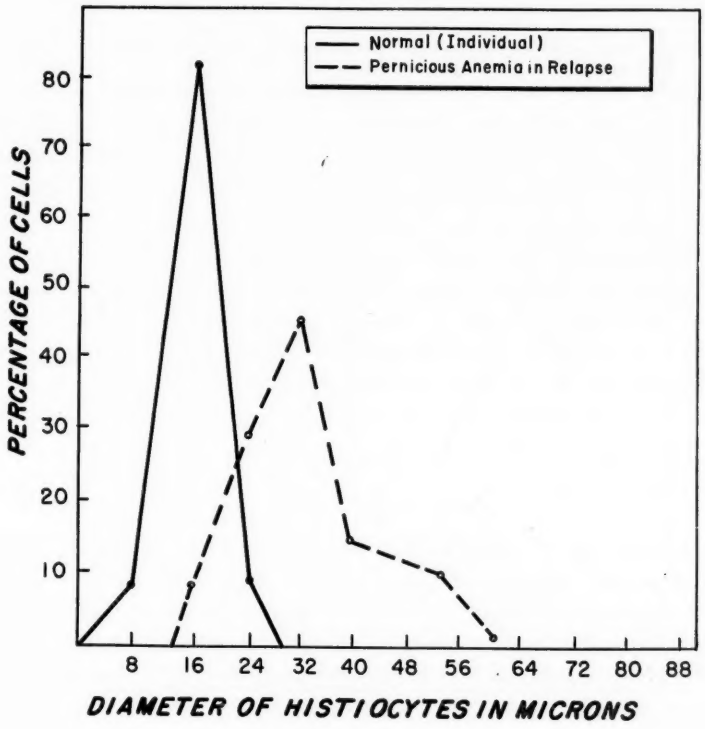


Fig. 8.—Typical normal phagocytic histiocytes of sputum seen in gastric secretion.
Fig. 9.—Enlarged atypical phagocytic histiocytes seen in untreated pernicious anemia.

remission. Any cell showing variation such as increase in size, multiple nuclei, bizarre chromatin arrangement was counted as being abnormal. The percentage of abnormal cells was determined and, in Chart V, is plotted against the grams per cent of hemoglobin. The patients with pernicious anemia in relapse had from 14 to 53 per cent of abnormal cells. The patient with the least number of these abnormal cells was also the only patient in the untreated group with a hemoglobin over 10 Gm. per cent. The average number of abnormal cells encountered was 28 per cent.

The cases of remission had fewer of the abnormal cells, the average being 8 per cent. There were three cases with more than 15 per cent abnormal cells present, and in each of those cases the abnormality present was increased in size.

CHART IV



The line on the chart represents counts on three successive gastric aspirations on the same patient. The lowest point is the count before any therapy. The middle point is after two weeks of vitamin B_{12} therapy, and the top point is after four weeks. It is interesting that, as the hemoglobin rises, the number of abnormal cells decreases. The history of this case is as follows:

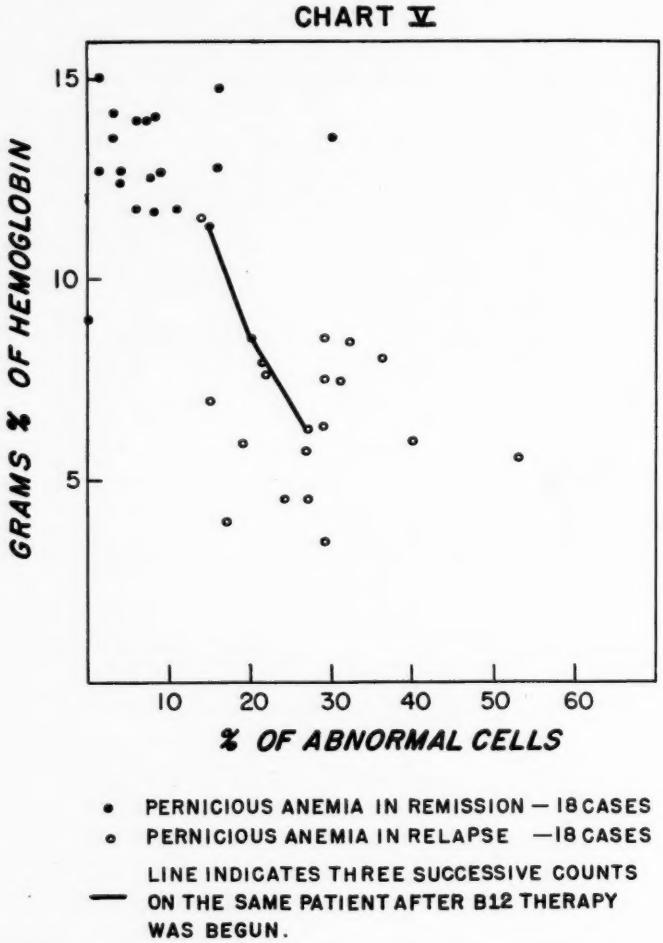
A 59-year-old housewife entered the hospital with a complaint of weakness of four months' duration. Laboratory data revealed a macrocytic normochromic anemia; red blood count of 1.67 million/c.mm., and a hemoglobin value of 6.3 Gm. per cent. Bone marrow study revealed 8 to 10 megaloblasts per high-power field.

The gastric smears of the four cases of severe iron deficiency anemia and the three cases of acute blood loss were examined for the presence of these abnormal features in the squamous epithelial cells. No such abnormalities were seen.

DISCUSSION

The important point in this study is whether or not these cellular abnormalities are directly dependent on the lack of the extrinsic factor. There are

several points to consider. Are these abnormalities due to the anemia itself? Though the hemoglobin values in the patients with acute blood loss and iron deficiency anemia were low and in the same range as those of the untreated pernicious anemia, no such abnormalities were found. A low hemoglobin of itself apparently does not produce this change in the cells.



A second factor which is present here is the absence of free hydrochloric acid in the gastric secretion after histamine. Since the patients in remission had no free acid, this factor can probably be disregarded as significant in the production of these distinctive cellular changes. In addition is the fact that many of the patients with gastric cancer have no free hydrochloric acid, but this type of cellular abnormality does not occur.

The evidence would seem to point to the lack of the extrinsic factor as the cause of the specific cellular changes. Since the size of squamous epithelial cells in the untreated group are much larger than normal, and since the size of such cells in cases of therapeutic remission approach normal, it would seem

to indicate that the extrinsic factor is at fault. Too, there is the evidence that few of the nuclear abnormalities are found in the cases in remission. The disappearance of the abnormal forms after therapy suggests the specific lack of the extrinsic factor as determining their presence.

Though this study has been limited to the cellular picture of the gastric secretion in pernicious anemia, these specific abnormal forms have been seen in sputum, in smears from the oral cavity, and in one instance in the vaginal smear.

The two common types of epithelium, squamous and columnar, have been studied. Both show specific abnormalities. In addition, phagocytic histiocytes show marked size increase in untreated pernicious anemia. These abnormal features of three distinct types of cells are comparable to the abnormal size of the cells in blood and bone marrow. Nucleated red cells encountered in pernicious anemia are considered to have an abnormal distribution of chromatin and abnormal mitoses. In addition, the enlarged neutrophil seen in Addisonian anemia and the abnormal megakaryocytes reported by Epstein[1] are examples of two other types of cells where the extrinsic factor seems to be essential for normal development. The same factor necessary for normal maturation of red blood cells appears to be essential for the maturation of other types of cells, such as the squamous epithelial cell, columnar cell, and histiocyte.

SUMMARY

Specific cellular changes in both squamous and columnar epithelial cells in untreated pernicious anemia have been described. These changes are almost completely absent in the patient in therapeutic remission. The suggestion is made that the extrinsic factor may be responsible for the normal maturation of epithelial cells just as it is for the normal maturation of the red blood cells.

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OPTICAL ISOMERS OF CYSTEINE IN THE PREVENTION OF LEUKOPENIA INDUCED BY NITROGEN MUSTARD

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ADMINISTRATION of L-cysteine to rabbits prior to injecting nitrogen mustard (HN2) partially prevents the severe leukopenia characteristically induced by HN2.[1] No such protective effect occurs if L-cysteine is administered at any time after the injection of HN2. Certain structural components appear to be essential for this protective effect. Thus many highly reactive compounds with structures closely related to L-cysteine fail to modify the leukopenia induced by HN2, and of those compounds studied, only those containing a sulfhydryl, amino, and carboxyl group in close apposition are effective.[2] Compounds in which there is substitution or alteration of any of these reactive groups have no demonstrable effect in preventing HN2-induced leukopenia. Neither the reducing action of a compound nor the presence by themselves of either free sulfhydryl, amino, or carboxyl groups can be correlated with the ability of a compound to prevent leukopenia when injected prior to HN2.

The mechanism by which L-cysteine exerts a protective effect is not known. The structural specificity required for this effect suggests that L-cysteine may protect some substance or substances essential for leukopoiesis from destruction by competitive inhibition of HN2. It is possible, however, that L-cysteine may prevent leukopenia by chemical inactivation of HN2 in vivo and that its effectiveness is due to availability at the site of damage by virtue of its structure.

In the following report, the specificity as well as the mechanism of L-cysteine protection was further investigated by comparing the relative effectiveness of D-cysteine and L-cysteine on the leukopenia induced by HN2. This study indicates that D-cysteine is not as effective as L-cysteine in preventing leukopenia. Therefore it is unlikely that L-cysteine protection is due to chemical inactivation of HN2.

METHODS

As in previous experiments,[1, 2] the protective effect of D-cysteine and L-cysteine against leukopenia induced by HN2 was studied in rabbits. Because of the difficulty in obtaining sufficent quantities of D-cysteine, its protective

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effect was studied in only 9 rabbits. The protective effect of D-cysteine was then compared with that of L-cysteine in 30 rabbits and with the effect of HN2 alone in 30 rabbits.

These studies were repeated in rats because of the relatively smaller amounts of D-cysteine required. Groups of 25 rats each were used to compare the effect of D-cysteine on HN2-induced leukopenia with that of L-cysteine and with the effect of HN2 alone.

Both D-cysteine hydrochloride* and L-cysteine hydrochloride† were freshly prepared as a 20 per cent solution in distilled water and the pH adjusted to 7.0 with 30 per cent sodium hydroxide; 600 mg. per kilogram of either D-cysteine or L-cysteine hydrochloride were injected intravenously in rabbits and intraperitoneally in rats prior to giving HN2.

A freshly prepared saline solution of HN2 (methyl bis [beta chlorethyl] amine hydrochloride)† containing 1 mg. per milliliter was used in each instance; 2.5 mg. of HN2 per kilogram of body weight were injected intravenously in rabbits ten minutes after the administration of either D or L-cysteine hydrochloride. Because of the sensitivity of rats to HN2, only 1.5 mg. of HN2 per kilogram of body weight were injected intravenously in these animals.

Daily total leukocyte and differential counts were obtained in duplicate. The protective effect of D-cysteine hydrochloride was determined by comparing the fall in these counts with those obtained when L-cysteine hydrochloride was administered prior to HN2, as well as when HN2 was given without cysteine.

RESULTS

1. *The Effect of D-cysteine and L-cysteine on Leukopenia Induced by HN2 in Rabbits.*—Following the injection of HN2, the maximum leukopenia and neutropenia invariably developed on the fourth or fifth day. When D-cysteine or L-cysteine was injected prior to the HN2, the maximum fall in the total leukocyte and absolute neutrophil count also developed on the fourth or fifth day (Figs. 1 and 2). The leukocyte and neutrophil counts obtained on the fourth day were therefore used to summarize the changes observed (Tables I and II).

In 30 control rabbits who received 2.5 mg. HN2 per kilogram without cysteine, the average total leukocyte count fell 79.7 per cent in four days (Table I and Fig. 1). The average initial count was 7,567 cells per cubic millimeter and the average count on the fourth day was 1,533 (standard error of the mean $[\sigma / n] = 172$). The average neutrophil count in these rabbits fell

*D-cysteine hydrochloride was obtained from the California Foundation for Biochemical Research, Los Angeles, Calif., and showed a specific rotation $[\alpha]_D = -3.4°$ in water. D-cysteine was also obtained from H&M Chemical Company, Ltd., Santa Monica, Calif., Lot No. 116-17-30.

†The L-cysteine hydrochloride and the nitrogen mustard used in these experiments were furnished through the courtesy of Merck & Co., Inc., Rahway, N. J.

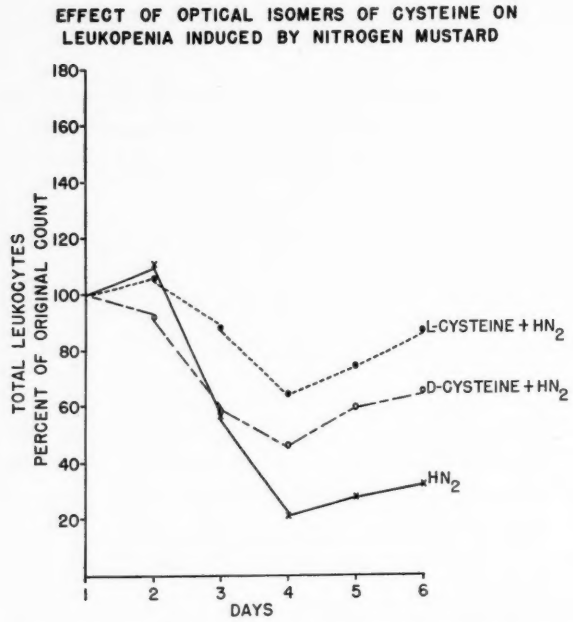

Fig. 1.—The administration of L-cysteine to rabbits prior to the injection of HN2 results in a significantly greater protection against leukopenia than that furnished by D-cysteine.

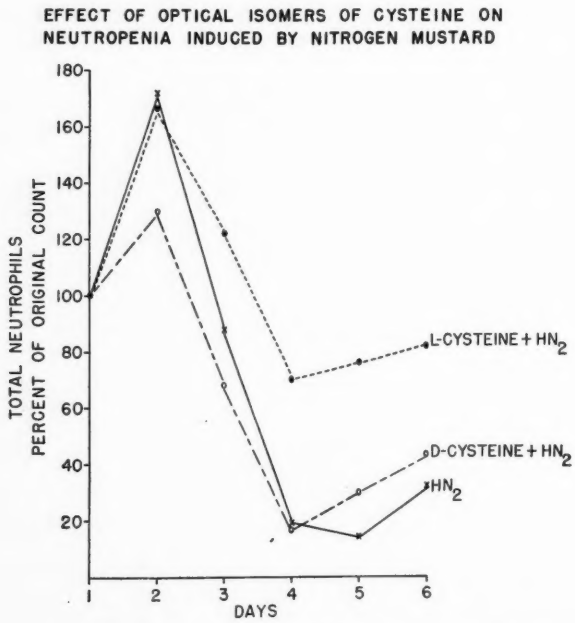

Fig. 2.—The administration of L-cysteine to rabbits prior to the injection of HN2 results in a significant modification of neutropenia. There is no significant difference between the neutropenia developing when D-cysteine is administered prior to HN2 and when HN2 is given without cysteine.

TABLE I. COMPARATIVE EFFECT OF D-CYSTEINE AND L-CYSTEINE ON LEUKOPENIA INDUCED BY NITROGEN MUSTARD IN RABBITS

	NO. OF RABBITS	AVERAGE INITIAL COUNT (TOTAL WBC'S)	AVERAGE LEUKOPENIA*		PER CENT FALL IN WBC'S†
			TOTAL WBC'S	STANDARD ERROR OF MEAN (σ / N)	
Nitrogen Mustard (HN2) 2.5 mg./kg.	30	7,567	1,533	172	79.7
L-Cysteine Prior To HN2	30	7,627	4,880	313	35.9
D-Cysteine Prior To HN2	9	9,189	3,533	281	61.6

*Counts taken on the fourth day after injecting HN2.

†Per cent fall = $\frac{\text{Initial Count} - \text{Count on 4th Day}}{\text{Initial Count}} \times 100.$

TABLE II. COMPARATIVE EFFECT OF D-CYSTEINE AND L-CYSTEINE ON NEUTROPENIA INDUCED BY NITROGEN MUSTARD IN RABBITS

	NO. OF RABBITS	AVERAGE INITIAL COUNT (TOTAL PMN'S)	AVERAGE NEUTROPENIA*		PER CENT FALL IN PMN'S†
			TOTAL PMN'S	STANDARD ERROR OF MEAN (σ / N)	
Nitrogen Mustard (HN2) 2.5 mg./kg.	30	2,807	567	108	79.8
L-Cysteine Prior To HN2	30	2,890	1,820	187	37.3
D-Cysteine Prior To HN2	9	4,567	789	156	82.8

*Counts taken on the fourth day after injecting HN2.

†Per cent fall = $\frac{\text{Initial Neutrophil Count} - \text{Neutrophil Count of 4th Day}}{\text{Initial Neutrophil Count}} \times 100.$

79.8 per cent in four days (Table II and Fig. 2). The average initial neutrophil count was 2,807 cells per cubic millimeter and the average neutrophil count on the fourth day was 567 cells per cubic millimeter (σ / n = 108).

When L-cysteine was administered prior to the HN2, the total leukocyte count and absolute neutrophil count fell 35.9 per cent and 37.3 per cent, respectively, in four days (Tables I and II, Figs. 1 and 2). The average initial leukocyte count was 7,627 cells per cubic millimeter and the average leukocyte count on the fourth day was 4,880 cells per cubic millimeter (σ / n = 313). The average initial neutrophil count was 2,890 cells per cubic millimeter and the average neutrophil count on the fourth day was 1,820 cells per cubic millimeter (σ / n = 187).

When D-cysteine was administered prior to the HN2, the total leukocyte count and absolute neutrophil count fell 61.6 per cent and 82.8 per cent, respectively, in four days (Tables I and II, Figs. 1 and 2). The average initial leukocyte count was 9,189 cells per cubic millimeter and the average leukocyte count on the fourth day was 3,533 cells per cubic millimeter (σ / n = 281). The average initial neutrophil count was 4,567 cells per cubic millimeter and the average neutrophil count on the fourth day was 789 cells per cubic millimeter (σ / n = 156).

Statistical analysis of the data reveals that the leukopenia developing when D-cysteine is administered is significantly greater than that developing when

L-cysteine is given prior to HN2. Thus there is no overlap in the confidence intervals ($\pm 2\sigma / n$) between the total leukocyte counts in these two groups and the P value is less than 0.01. When the counts are adjusted for the higher initial count in the animals receiving D-cysteine, P is still less than 0.01.

Analysis of the changes in the neutrophil counts reveals that there is no significant difference between the neutropenia developing when D-cysteine is administered prior to HN2 and when HN2 is used alone (P is greater than 0.01). There is, however, a significant protection of neutrophils when L-cysteine is administered prior to HN2.

2. *The Effect of D-cysteine and L-cysteine on Leukopenia Induced by HN2 in Rats.*—Following intravenous injection of 1.5 mg. of HN2 per kilogram in rats the maximum leukopenia also developed on the fourth or fifth day. When HN2 was given without cysteine the average fall in total leukocyte count on the fourth day was 89.3 per cent and the average fall in the absolute neutrophil count was 84.9 per cent of the initial value. When L-cysteine was injected prior to the HN2, the average fall in the total leukocyte and absolute neutrophil counts on the fourth day was 44.7 and 65.3 per cent, respectively. When D-cysteine was injected prior to the HN2, the average fall in total leukocyte and absolute neutrophil counts on the fourth day was 58.2 and 81.7 per cent, respectively (Table III).

TABLE III. COMPARATIVE EFFECT OF D-CYSTEINE AND L-CYSTEINE ON LEUKOPENIA AND NEUTROPENIA INDUCED BY NITROGEN MUSTARD IN RATS

	NO. OF RATS	AVERAGE LEUKOPENIA* (PER CENT†)	AVERAGE NEUTROPENIA* (PER CENT†)
Nitrogen Mustard (HN2) 1.5 mg./kg.	25	89.3	84.9
L-Cysteine Prior To HN2	25	44.7	65.3
D-Cysteine Prior To HN2	25	58.2	81.7

*Counts taken on the fourth day after injecting HN2.

†Per cent fall $= \frac{\text{Initial Count} - \text{Lowest Count}}{\text{Initial Count}} \times 100.$

DISCUSSION

Under the conditions of these experiments, equimolar amounts of D-cysteine are not as effective as L-cysteine in preventing the toxic effects of HN2 on leukocytes. In rabbits this is most apparent in the changes which take place in the neutrophils (Fig. 2 and Table II). The average maximum fall in neutrophils developing when L-cysteine was given was 30.2 per cent of the initial value compared with a fall of 81.9 per cent when D-cysteine was given and 86.1 per cent when HN2 was given without cysteine. The changes in the total leukocyte count are not as striking as the changes in neutrophils but significantly greater protection is obtained with L-cysteine than with D-cysteine.

Although L-cysteine was more effective than D-cysteine in modifying HN2-induced leukopenia and neutropenia in rats, the differences are not as marked as those in rabbits. It is possible either that the rat can utilize D-cysteine better than the rabbit or else that it can convert some of the D-cysteine to L-cysteine.

It should be noted also that the protective effect of L-cysteine in rats is not as marked as in rabbits. This may be due to a greater sensitivity of the rat to HN2. The majority of the cells in the peripheral blood of rats are lymphocytes and these cells are known to be more sensitive to HN2. Furthermore, smaller amounts of HN2 are lethal for rats than for rabbits. Thus only 1.5 mg. HN2 per kilogram could be used safely in rats compared with 2.5 mg. HN2 per kilogram in rabbits.

The failure of D-cysteine to exert as much protection as L-cysteine may be due to an inability on the part of leukocytes or their precursors to utilize the dextrorotatory isomer. It is also possible that D-cysteine may be selectively excreted or inactivated. Evidence, however, does exist that D-cysteine cannot be utilized to the same extent as L-cysteine. Thus, Stekol[3] has shown that D-cysteine cannot be used for growth in cysteine deficient animals whereas L-cysteine readily promotes growth. Similarly, experiments now in progress demonstrate that D-cysteine cannot be utilized in vitro by immature leukocytes. Thus when leukemic leukocytes are incubated with L-cysteine prior to adding isotopic L-cystine, there is a marked reduction in the uptake of the isotope compared with the uptake by cells which are incubated without L-cysteine. When these cells are incubated with D-cysteine prior to adding isotopic L-cystine, there is no diminution in the uptake of the isotope.[4] It is possible that protection against the leukotoxic effect of HN2 requires intracellular sparing of a substance or substances essential for leukocyte metabolism and that D-cysteine fails to protect because it cannot be utilized or is unavailable at an intracellular level to compete with HN2.

The data indicate that a specific spatial configuration in addition to the presence of vicinal sulfhydryl, amino, and carboxyl groups is required to prevent the damaging effect of HN2 on rabbit leukocytes. Therefore, it is unlikely that the protective effect of L-cysteine is due solely to chemical inactivation of HN2. The requirement of such a high degree of structural and spatial specificity in leukocyte protection suggests that L-cysteine or closely related compounds may have a unique role in leukocyte metabolism.

SUMMARY AND CONCLUSIONS

D-cysteine is not as effective as L-cysteine in preventing the leukopenia and neutropenia induced by HN2.

Thus a specific spatial configuration as well as the presence of a sulfhydryl, amino, and carboxyl group in close apposition appears to be required for preventing the leukotoxic effect of HN2.

It is unlikely that the protective effect of L-cysteine is due solely to chemical inactivation of HN2 in vivo.

The requirement of such structural and spatial specificity suggests that L-cysteine or closely related compounds may have a unique role in leukocyte metabolism.

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SEROLOGIC TESTS FOR SYPHILIS IN INFECTIOUS MONONUCLEOSIS

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IT IS recognized that infectious mononucleosis may at times cause nonspecific reactions in serologic tests for syphilis. Reports vary widely, however, as to the incidence of such reactions. Thus Gooding[1] encountered complement-fixing antibody in as many as 16 of 27 individuals with the disease, while Kaufman[2] found only 3 of 64 patients to react in the complement-fixation test; equally inconsistent results were obtained in the flocculation tests for syphilis. These and similar reports have been discussed in comprehensive reviews.[3-5] More recently Wechsler and his associates[6] performed Kahn tests on the sera of 265 individuals with infectious mononucleosis, and attributed 8 false reactions to the disorder. The explanation for these diverse experiences is not clear. If it is accepted that infectious mononucleosis is indeed infectious in origin, it may be that strains of the etiological agent differ in their capacity to elicit false serologic reactions. The role of this factor cannot be evaluated, however, until the etiology of the disease has been established. Other factors may contribute to the wide variation in serologic findings. These include differences in the specificity of the tests for syphilis, as well as variations in the frequency of blood sampling, or in the time of sampling with respect to stage of the disease.

An opportunity to study the influence of such factors upon nonspecific serologic activity arose during an extensive investigation of infectious mononucleosis.[7-9] Sera were available from a large group of patients for whom the diagnosis had been established unequivocally on clinical evidence and laboratory tests. Each of these sera was subjected to eight different serologic tests for syphilis. The results form the basis for the present report.

MATERIALS AND METHODS

Sera.—The 594 specimens of serum tested in this study were obtained from 183 college students and other young adults admitted to the University of Michigan Student Health Service and the University Hospital; examination revealed no clinical, anamnestic, or previous serologic evidence of treponematosis. Of this patient group 110 were men, and 73 were women. The diagnosis of infectious mononucleosis in each instance was based upon clinical findings supported by the results of hematologic and serologic (heterophil agglutination) tests. The differential counts of leukocytes were made from cover-slip preparations. The heterophil agglutination tests were performed according to a technique described elsewhere,[7, 8] all sera from a given individual being examined at one time with erythrocytes from the same sheep. The collected specimens were finally shipped by air-courier to Washington, D. C., where the serologic tests for syphilis were performed.

Serologic Tests for Syphilis.—Each of the 594 sera was subjected to eight different tests for syphilis at the Department of Serology, Army Medical Service Graduate School. The tests,

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TABLE I. SEROLOGIC TESTS FOR SYPHILIS USED IN THIS STUDY

TYPE OF TEST	NAME AND ABBREVIATION		ANTIGEN	REFERENCES
Slide flocculation	VDRL	(VDRL)	Cardiolipin	(10, 11)
	Kline	(Kli)	Cardiolipin	(12)
	Rein-Bossak	(R-B)	Cardiolipin	(13)
	Mazzini	(Maz)	Mazzini	(14)
Tube flocculation	Kahn standard	(Kahn)	Kahn	(15)
Complement-Fixation	Kolmer	(Kol)	Kolmer	(16)
	Kolmer	(KolC)	Cardiolipin	(17, 18)
	CF48	(CF48)	Cardiolipin	

with the abbreviations used to simplify tabulation, are listed in Table I. Details of the procedures and their interpretation are to be found in the given references; concise directions for most of the techniques have been collected in a manual of serologic tests for syphilis.[19] Of the eight tests, five employed cardiolipin antigens; these antigens were compounded from cardiolipin and lecithin prepared by the Department of Biochemistry, Army Medical Service Graduate School, and shown to conform chemically and serologically with standards of the Division of Laboratories and Research, New York State Department of Health. The Mazzini, Kahn, and Kolmer antigens were purchased from commercial sources, and were from lots approved by the author serologists.

The CF48 test was an experimental complement-fixation procedure based essentially upon reference,[20] and under evaluation currently (1948) as a diagnostic aid. The antigen was an ethanol solution containing cardiolipin 0.0175 per cent, lecithin 0.0875 per cent, and cholesterol 0.30 per cent[21]; it was identical with that employed in the Kolmer (KolC) procedure. Standard volume for the CF48 test was 1.5 ml., 0.3 ml. aliquot volumes being allotted the five essential reagents. In practice 0.1 ml. of sera plus 0.2 ml. of salt solution was incubated 16-18 hours at 3-6° C. with 0.3 ml. each of complement (three 50 per cent units) and an optimal dilution (1:250) of the antigen. Sensitized erythrocytes (0.6 ml.) were then added, and the tests read after an additional 30 minutes at 37° C. A detailed account of the method at a later stage of development has been presented in connection with comprehensive studies of sporotrichosis.[22]

To conserve serum the Kolmer complement-fixation tests were carried out with one-fifth volumes of the reagents,[16] and the CF48 test with one-half volumes. To minimize errors of measurement under the conditions sera were delivered accurately from 0.1 ml. pipettes, and the other reagents added with precisely standardized automatic pipetting machines.* Results of the complement-fixation tests were read by comparison with standards representing the percentages of hemolysis at 10 per cent intervals from 0 to 100 per cent. To avoid artificial differences in reactivity all sera from a given patient were examined on one occasion with the same reagents. Apparent technical inaccuracies were corrected by repeating the tests. Single unsatisfactory or insufficient specimens from five persons accounted for nine test omissions, one of the omissions (Table III, Patient 90) occurring in a case where concurrent results suggested that a reaction might have been observed.

RESULTS

Table II indicates the frequency with which nonspecifically reactive individuals were encountered in the different tests for syphilis; a total of 4,743 serologic examinations are represented. Of the 183 patients with infectious

*Brewer Automatic Pipetting Machine, Model 120, Baltimore Biological Laboratories, Inc., Baltimore, Md.

mononucleosis 13 (7.1 per cent) reacted in one or more tests at some time during the course of their illness. Two of this group (1.1 per cent) reacted in the slide flocculation tests. Ten (5.5 per cent) were found to react in the tube flocculation test, although six of their number showed only doubtful reactions. Four persons (2.2 per cent) yielded nonspecific reactions in the Kolmer complement-fixation tests, and two of these (1.1 per cent) reacted in the experimental complement-fixation procedure (CF48).

TABLE II. NUMBERS OF INDIVIDUALS WITH INFECTIOUS MONONUCLEOSIS EXHIBITING NONSPECIFIC REACTIVITY IN SEROLOGIC TESTS FOR SYPHILIS; RESULTS OBTAINED WITH 594 SERA FROM 183 PATIENTS

SEROLOGIC DEGREE OF REACTION	INDIVIDUALS REACTING IN THE GIVEN SEROLOGIC TEST*							
	SLIDE FLOCCULATION				TUBE FLOCCULATION	COMPLEMENT-FIXATION		
	VDRL (C)	Kli (C)	R-B (C)	Maz	KAHN	Kol	KolC (C)	CF48 (C)
Positive	2 (1.1%)	2 (1.1%)	2 (1.1%)	2 (1.1%)	4 (2.2%)	4 (2.2%)	4 (2.2%)	2 (1.1%)
Doubtful	0	0	0	0	6 (3.3%)	0	0	0
Negative	181	181	181	181	173	179	179	181

*The tests represented are the VDRL, Kline (Kli), Rein-Bossak (R-B), Mazzini (Maz), Kahn Standard (Kahn), Kolmer (Kol), Kolmer Cardiolipin (KolC), and CF48; those employing cardiolipin antigens are further identified by the subscript (C).

Table III gives detailed serologic results for the thirteen individuals who exhibited false reactions; the findings are located chronologically with respect to the day of disease. Included for reference are titers obtained with the same sera in heterophil agglutination tests.

DISCUSSION

The number of patients with infectious mononucleosis found to react nonspecifically in serologic tests for syphilis was lower in the present experience than would have been anticipated from previous reports; in no test (Table II) did the number of individuals yielding positive reactions exceed 2.2 per cent. The most remarkable feature of the observations was the fact that no one patient reacted in all eight tests for syphilis, or even concurrently in the three types of test, i.e., slide flocculation, tube flocculation, and complement fixation. One person (Table III, Patient 94) exhibited serologic activity, although not concurrently, in seven of the tests. It had been hoped that an extensive study such as this would afford some basis for conclusions regarding the relative specificity of the tests for syphilis. This proved not to be the case, however; the nonspecific reactions, besides being few in number, appeared randomly distributed among the several procedures. This was in marked contrast to the decisive evidence obtained in similar studies of malaria.[23, 24] In both cited experiences the frequency of false reactions was high, with the great majority occurring in tests which employed standard alcoholic tissue extracts as antigens; relatively few nonspecific results were encountered in the tests with cardiolipin antigens.

TABLE III. DETAIL OF THE SEROLOGIC FINDINGS FOR THIRTEEN PATIENTS WITH INFECTIOUS MONONUCLEOSIS WHO REACTED NONSPECIFICALLY IN TESTS FOR SYPHILIS

PATIENT NUMBER	DAY OF DISEASE	TITER* IN HETEROPHIL AGGLUTINATION TEST	RESULTS OF SEROLOGIC TESTS FOR SYPHILIS†							
			SLIDE FLOCCULATION				TUBE FLOCCULATION KAHN	COMPLEMENT-FIXATION		
			VDRL (C)	Kli (C)	R-B (C)	Maz		Kol	Kolc (C)	CF48 (C)
16	8	256	–	–	–	–	–	–	–	–
	14	2,048	–	–	–	–	–	P	P	P
	17	2,048	–	–	–	–	–	P	P	P
45	5	8	–	–	–	–	–	–	–	–
	13	256	–	–	–	–	P	P	P	–
	19	1,024	–	–	–	–	P	P	P	–
	27	1,024	–	–	–	–	P	–	–	–
	32	512	–	–	–	–	P	–	–	–
	117	64	–	–	–	–	–	–	–	–
50	4	64	–	–	–	–	–	–	–	–
	10	256	–	–	–	–	–	–	–	–
	15	512	–	–	–	–	D	–	–	–
	22	256	–	–	–	–	–	–	–	–
	36	128	–	–	–	–	–	–	–	–
	53	64	–	–	–	–	–	–	–	–
	67	64	–	–	–	–	–	–	–	–
	82	64	–	–	–	–	–	–	–	–
	120	64	–	–	–	–	–	–	–	–
61	35	64	–	–	–	–	D	–	–	–
	49	256	–	–	–	–	–	–	–	–
	63	128	–	–	–	–	–	–	–	–
	79	128	–	–	–	–	–	–	–	–
	97	128	–	–	–	–	–	–	–	–
	112	128	–	–	–	–	–	–	–	
	126	128	–	–	–	–	–	–	–	
88	9	512	–	–	–	–	P		–	
90	9	2,048	P	P	P	P	Insuf.	–	–	–
	273	0	–	–	–	–	–	–	–	–
94	2	256	–	–	–	–	–	P	P	–
	7	1,024	P	P	P	P	P	–	–	–
	18	512	P	P	P	P	P	–	–	–
	36	256	–	–	–	–	–	–	–	–
	200	16	–	–	–	–	–	–	–	–
112	14	512	–	–	–	–	D	–	–	–
	161	32	–	–	–	–	–	–	–	–
137	7	512	–	–	–	–	–	–	–	–
	15	4,096	–	–	–	–	P	–	–	–
	20	4,096	–	–	–	–	–	–	–	–
	27	2,048	–	–	–	–	–	–	–	–
	34	1,024	–	–	–	–	–	–	–	–
	48	512	–	–	–	–	–	–	–	–
	56	512	–	–	–	–	–	–	–	–
	63	128	–	–	–	–	–	–	–	–
	70	128	–	–	–	–	–	–	–	–
	77	128	–	–	–	–	–	–	–	–
	84	64	–	–	–	–	–	–	–	–
	90	64	–	–	–	–	–	–	–	–
	98	64	–	–	–	–	–	–	–	–
	103	64	–	–	–	–	–	–	–	–
162	6	512	–	–	–	–	D	–	–	–

TABLE III—CONT'D

PATIENT NUMBER	DAY OF DISEASE	TITER* IN HETEROPHIL AGGLUTINATION TEST	RESULTS OF SEROLOGIC TESTS FOR SYPHILIS†							
			SLIDE FLOCCULATION				TUBE FLOCCULATION KAHN	COMPLEMENT-FIXATION		
			VDRL (C)	Kli (C)	R-B (C)	Maz		Kol	Kolc (C)	CF48 (C)
165	4	32	–	–	–	–	–	P	P	P
	6	64	–	–	–	–	–	P	P	P
	10	64	–	–	–	–	–	–	–	–
	18	64	–	–	–	–	–	–	–	–
	24	32	–	–	–	–	–	–	–	–
	40	32	–	–	–	–	–	–	–	–
	79	16	–	–	–	–	–	–	–	–
	152	16	–	–	–	–	–	–	–	–
174	28	1,024	–	–	–	–	D	–	–	–
	49	256	–	–	–	–	D	–	–	–
	61	128	–	–	–	–	D	–	–	–
176	(18)‡	32	–	–	–	–	–	–	–	–
	(25)	32	–	–	–	–	–	–	–	–
	(28)	32	–	–	–	–	D	–	–	–
	21	32	–	–	–	–	–	–	–	–
	26	128	–	–	–	–	D	–	–	–
	34	256	–	–	–	–	D	–	–	–
	43	128	–	–	–	–	D	–	–	–

*Reciprocal of the highest serum dilution yielding a significant (2-plus) reaction.

†Results are designated P (positive), D (doubtful), – (negative), or Insuf. (insufficient); the key to test abbreviations appears as a footnote to Table II.

‡Parentheses denote 1947 illness clinically similar to infectious mononucleosis, but without typical changes in the titer of heterophil agglutinins or in leukocytes; illness occurring six months later (1948) filled all criteria required for the diagnosis.

The low incidence of serologic reactions and temporal vagaries in blood sampling made it impossible in the present study to estimate the relative time of appearance of serologic activity or its duration in the different tests. Most of the patients were college students, and the collection of specimens was at times delayed or prevented by the advent of vacation or graduation. As a consequence some critical samples were missed, or a follow-up specimen secured at a time possibly long after the individual had reverted to serologically negative status. It was clear, however, that the nonspecific serologic response in infectious mononucleosis is transitory in nature, and falls properly in the group of "acute" biologic false reactions.[3-5]

SUMMARY AND CONCLUSIONS

Eight serologic tests for syphilis were evaluated with respect to the frequency of nonspecific reactions due to infectious mononucleosis; 594 sera from 183 patients were examined.

Thirteen (7.1 per cent) of the patients exhibited transient nonspecific serologic reactions in one or more tests for syphilis at some time during their illness. No patient, however, reacted in all eight tests, or even concurrently in all three types of test. Thus, two individuals (1.1 per cent) showed positive reactions in slide flocculation tests (VDRL, Kline, Rein-Bossak, and Mazzini). Four patients (2.2 per cent) were positive, and six (3.3 per cent) yielded doubtful reactions in a tube flocculation test (Kahn standard). Four persons (2.2 per

cent) exhibited positive reactions in complement-fixation tests (Kolmer) with standard and cardiolipin antigens, and two of these (1.1 per cent) were positive also in an experimental complement-fixation procedure (CF48).

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PRECIPITINS IN HUMAN HISTOPLASMOSIS

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SEROLOGIC tests as aids in the diagnosis of histoplasmosis have been of three types: (a) complement-fixation, (b) collodion or red-cell agglutination, and (c) precipitin. The complement-fixation test is the one most extensively used. Fixation of complement was obtained[1, 2] employing the whole cells of the yeastlike phase as antigen, with sera from immunized animals, experimentally infected animals, and naturally infected human beings. Maximum titers in infected rabbits developed during the fourth to fifth week after inoculation, with positive titers persisting up to six months postinoculation.[2] Antigens prepared by grinding the yeastlike phase have also been used in complement-fixation tests with human and hyperimmune rabbit sera.[3] The filtrate from cultures of mycelial growth (histoplasmin) has fixed complement in the presence of sera from immunized rabbits,[2] experimentally infected guinea pigs, and human beings.[4, 5]

Collodion particles on which histoplasmin had been previously adsorbed agglutinated in the presence of antibody from hyperimmunized rabbits and from human beings with histoplasmosis.[6, 7] Sheep erythrocytes sensitized with histoplasmin also agglutinated when mixed with homologous antiserum from immunized rabbits.[8]

Sera of hyperimmune animals showed antibodies in a precipitin test in which fractions from the mycelial phase were employed as antigen, although the sera from experimentally infected rabbits were negative.[9] In another study on sera from experimentally infected rabbits, however, precipitins appeared during the second week after the administration of the infective dose, reached their peak titers about one week later, and finally disappeared about ten weeks after the day of original infection.[2] Histoplasmin was found to be the most active antigen of 4 tested.

In spite of the foregoing studies, difficulty has frequently been encountered in finding antibodies in cases of human histoplasmosis. This has been especially true in patients with few or mild clinical symptoms. The use of the precipitin test as an aid to diagnosis of histoplasmosis in human beings was suggested by the presence of precipitins for a short period of time during the early or acute phase of the disease in rabbits,[2] and by Smith's success in demonstrating the value of the precipitin test in the diagnosis of coccidioidomycosis.[12] The present report demonstrates (a) the occurrence of precipitins in patients with acute or chronic histoplasmosis and (b) the value of the precipitin test as an aid in the diagnosis of this disease.

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MATERIALS AND METHODS

The fungi in these studies were from the collection maintained at the Rocky Mountain Laboratory. The two isolates of *Histoplasma capsulatum* employed were No. 6515, originally obtained from a dog and used as the source of yeastlike-phase antigens, and No. 6510, originally obtained from a person who died of histoplasmosis. The broth filtrate from the mycelial growth was used as the precipitin antigen and was obtained by growing isolate No. 6510 in 100 ml. of asparagine glucose medium[10] in 250 ml. flasks for sixty to ninety days, at room temperature, and then by filtering the broth through a Berkefeld N. filter. Heat-killed (one hour at 56° C.) whole cells of the yeastlike phase which were grown in a casamino dextrose broth* under constant rotation[11] at 37° C. for four to five days were used as the antigen in the complement-fixation tests.

Both complement-fixation and precipitation tests were conducted on each serum examined. The complement-fixation test was carried out as previously described,[1] with 2 units of hemolysin, 2 units of complement, and the amount of antigen that gave the highest complement-fixation titer with a serum of known capacity. This amount of antigen had been determined to be 8×10^8 formalin-killed cells per milliliter of the yeastlike phase of isolate 6515. The mixture of serum, antigen, and complement plus the necessary serum, antigen, complement, and cell controls was incubated for one hour at 37° C. The addition of sensitized sheep cells was followed by incubation for another hour at 37° C., with final readings made after overnight refrigeration. One hundred per cent end points were used throughout.

In the precipitation test, equal quantities of antiserum and dilutions of antigen were drawn into 3-inch lengths of capillary tubing, incubated for two hours at 37° C., and refrigerated for thirty-six hours at 3° to 5° C. A control of uninoculated asparagine-glucose medium was included in each test. One lot of antigen was used throughout the present study.

It is fully realized that only by carrying on precipitations at the equivalence zone can the exact amount of antibody in a given serum be determined. However, for screening sera against histoplasmosis by precipitation techniques it would be impractical to conduct optimal proportions on each serum. Preliminary tests indicated that the antigen-dilution technique would give the information desired.

Since precipitins were detectable in the sera of some human beings and animals for only short periods of time, the possibility presented itself that an excess of antibody was inhibiting precipitation. Dilution of such sera failed, however, to indicate the continued presence of precipitins. Also, dilution of sera which had high or low titers of complement-fixing antibodies and which had no demonstrable precipitins by the antigen-dilution method failed to reveal additional precipitating antibodies. Only very rarely has an "inhibition zone" been observed in the high concentrations of antigen.

RESULTS

One of the better methods for determining the humoral antibody response of a host to an organism is to obtain a series of serum samples early and late in the course of illness. This ideal situation, however, is not always possible to attain with mild cases of histoplasmosis because of the difficulties involved in the early diagnosis of the disease. Eight cases are here presented to illustrate the usefulness of the precipitin test as an aid in diagnosis.†

	(wt. in grams)
*Difco casamino acids (technical)	10.0
Dextrose	3.0
Yeast-extract dialysate, equivalent of	3.0
Sodium chloride	2.5
Cysteine hydrochloride	0.5
Potassium chloride	2.5
Disodium phosphate	4.0
Distilled water to make	1,000 ml.

†A statistical analysis of the value of the precipitation and complement-fixation techniques in the diagnosis of human histoplasmosis is being reported elsewhere.[13]

These 8 cases may be divided into two categories: the first consists of 6 *acute* cases (patients I to VI), of which 2 were proved by the recovery of the organisms in culture, while the other 4 were among persons infected in the laboratory as evidenced by change from a negative to a positive histoplasmin skin test and the development of lung lesions demonstrable by x-ray in 3 of the 4 individuals; the second category consists of 2 *chronic* cases (patients VII and VIII) of which one was proved by culture, while the other had a clinical history and x-ray findings compatible with histoplasmosis even though cultures of *H. capsulatum* were not isolated. One of the 3 patients with histoplasmosis proved by cultures (patient VII) is seriously ill, tuberculosis having been demonstrated as a complication of histoplasmosis. The other in the second category (patient VIII) has serious and permanent lung changes, which are, however, not severe enough to require hospitalization.

CLINICAL STUDIES

Proved Histoplasmosis With Sarcoidosis and Ocular Lesions: Improved.—

CASE 1.—The patient (G. W.), a Negro woman, aged 27 years, had lived in Texas until 1949, when she came to Kansas City. She was studied because of miliary lung lesions noted by x-ray.

History: The patient complained of redness of the right eye beginning in July, 1951. Since September, 1951, she had had blurring of vision, at which time generalized enlargement of the lymph nodes was also noted. In November, biopsy of the left inguinal lymph node led to a diagnosis of sarcoidosis. During the winter of 1951, the patient developed a chest cold with cough productive of brownish sputum but no blood. This continued to the present. Occasional left pleuritic pain had been noted. In February, 1952, there had been gradual failing of vision in the left eye. About this time night sweats had occurred occasionally. A diagnosis of nodular iritis had been made just before admission to the hospital.

Admission findings: On March 28, 1952, physical examination revealed the presence of nodular iritis in both eyes, palpable liver and spleen, generalized lymph node enlargement, and a diffuse mass, measuring 3 by 4 cm. in area, in the right triceps muscle. Some dullness but no râles were noticed in the right upper and middle and the left lower chest. Red blood cell count was 3.9 million, hemoglobin 65 per cent, white blood cell count 6,250, polymorphonuclear leukocytes 68 per cent, lymphocytes 22 per cent, eosinophils 4 per cent, and monocytes 6 per cent. The Kahn test was doubtful; Wassermann was negative. Total serum protein was 8.8; globulin 4.72; albumin 4.08. The sedimentation rate, on March 30, was 24 mm. in 60 minutes. X-ray of the chest showed disseminated soft irregular lesions, peribronchial in character, in both lungs. There was acute pleurisy at the right base and some pleurisy along the left lateral chest. Annular shadows suggesting cavities were noted in both left and right upper lung fields. X-rays of the hands and feet were negative.

Skin tests: Tuberculin, coccidioidin, and blastomycin skin tests were each negative on three occasions (Table I). The histoplasmin test was negative at first, but later became positive.

Mycology and bacteriology: Nine sputa were negative for tubercle bacilli and fungi by culture and 4 by mouse inoculation. Lymph node biopsy and bone marrow cultures were negative for tubercle bacilli and fungi. Two of three gastric aspirations were positive for *H. capsulatum* by mouse inoculation.

Course: The admission diagnosis was sarcoidosis with ocular lesions. The temperature ranged from 100 to 101° F. irregularly for the first nineteen days, but for the last nine days no elevation of temperature was noted. On April 15, 1952, therapy was begun

TABLE I. SUMMARY OF LABORATORY AND CLINICAL FINDINGS

CASE	FIRST OBSERVATION	SKIN TEST* DATE	H	T	B	C	MYCOLOGY & BACTERIOLOGY DATE	SPECIMEN	TEST	H.c.†	T.b.†	CHEST X-RAY	STATUS	ADDITIONAL FINDINGS
I. G.W., woman, 27 years	3/12/52	3/12/52	-	-	-	-	4/ 3/52	Lymph node	Culture	Negative	Negative	3/12/52 miliary infiltration thin-walled cavity L.3.‡ Pleurisy right base. 10/19/52 slight clearing.	Improving 10/28/52	Nodular iritis in both eyes. Muscle biopsy-sarcoid. Biopsy inguinal node-sarcoid. Blood proteins 8.8, globulin 4.72, albumin 4.08.
		3/26/52	+	-	-	-								
		4/ 1/52	+	-	-	-	4/ 3/52	Bone marrow	Culture	Negative	Negative			
							3/29/52 to 4/ 9/52	9 sputa	Culture	Negative	Negative			
							4/9, 4/10 6/17, 6/18	4 sputa	Mice	Negative				
							4/4, 4/7, 4/8	3 gastric	Mice	2 positive				
II. P. A., man, 28 years	2/ 4/52	2/ 4/52	-	+	-	-	3/ 8/52	8 sputa	Culture	Negative		2/5/52 negative. 3/4/52 infiltration R.4 & L.3 with node. 3/18/52 clearing. 4/9/52 L. worse. 5/2/52 some clearing. 9/20/52 lung clear, node large.	Well 11/1/52	3/4/52 heterophile titer 1:64 3/10/52 heterophile titer 1:32
		2/ 6/52	-				3/9, 3/10							
							3/11		Mice	Negative				
		2/19/52	-				3/12							
		3/ 3/52	+				3/13							
		10/ 8/52	+	+	+	-	3/14, 3/19							
III. A. W., woman, 49 years	4/ 3/51	4/ 3/51	-	+				No sputa				4/3/51 to 9/4/51 negative. 11/21/51 pneumonic area R.3-4. 4/25/52 clearing. 5/15/52 negative	Well 11/1/52	
		4/16/51	-											
		5/21/51	+											
IV. F. B., man, 64 years	4/10/51	4/10/51	-	+			4/26/52	4 sputa	Culture	Negative		Five x-rays from 4/18/51 to 8/1/52 show only pulmonary fibrosis	Well 11/1/52	6/19/52 sed. rate 14 mm. 60 min. 7/25/52 sed. rate 15 mm. 60 min. 8/11/52 sed. rate 20 mm. 60 min.
		4/18/51	-											
		6/11/51	-				5/15/52		Mice	Negative				
		8/20/51	-				5/16/52							
		10/24/51	-				5/26/52							
		11/19/51	-											
		1/ 2/52	-											
		2/20/52	-											
		3/19/52	-											
		4/ 2/52	-	(+ at 5 days)										
		4/22/52	+											
		10/ 8/52	+	+	-	-								
V. H. N., man, 27 years	3/19/52	3/19/52	+	+	+	-		No sputa				1/30/51 neg. 3/31/52 infiltration R.2. 8/11/52 clear-	Well 11/1/52	6/19/52 sed. rate 14 mm. 60 min.

				4/22/52	+							
				10/ 8/52	+	+	-	-				
V.	H. N., man, 27 years	3/19/52	3/19/52	+	+	+	-	No sputa				1/30/51 neg. 3/31/52 infiltration R.2. 8/11/52 clearing.	Well 11/1/52	6/19/52 sed. rate 14 mm. 60 min. 7/25/52 sed. rate 15 mm. 60 min. 8/11/52 sed. rate 20 mm. 60 min.
VI.	U. C., man, 50 years	12/ 5/51	12/ 5/51	+	-	-	+	12/10/51	Gastric	Culture		Negative	12/5/51 fibrosis both apices. 9/3/52 may be a little worse.	Working 10/16/52	Choreoretinitis right eye.
			1/30/52	+	-	-	-	12/11/51	Sputum	Smear		Negative			
								12/11/51	Gastric	Culture	Negative	Negative			
										Mice	Negative				
								12/12/51	Gastric	Culture	Negative	Negative			
										Mice	Negative				
								2/29/52	Gastric	Culture	Negative				
										Mice	Negative				
								3/10/52	Gastric	Culture	Negative				
										Mice	Positive				
								3/11/52	Gastric	Culture	Negative				
										Mice	Positive				
VII.	P. C., woman, 29 years	11/ 1/51	11/19/51	+	+	-	-	11/17/51	10 sputa	Smear		8 negative		11/1/52 in tuberculosis hospital under treatment	
			5/28/52	+	+	-	-			Culture		4 negative			
								to	3 sputa	Culture	2 positive				
										Mice	2 positive				
									6 sputa	Smear		6 negative			
										Culture		1 negative			
								1/18/52	2 lung	Culture	1 positive				
										Mice	2 positive				
									3 pleural Fluid	Culture	2 positive				
										Mice	3 positive				
								5/20/52 to 5/23/52	5 sputa	Smear		5 positive			
										Culture		5 positive			
									2 sputa	Mice	2 negative				
								6/17/52	Sputum	Culture		Positive			
								8/19/52	Sputum	Culture		Positive			
								10/24/52	Sputum	Culture		Negative			
VIII.	R. H., man, 44 years	2/ 4/52	2/ 4/52	-	-	-	-	2/ 5/52	Sputum	Culture	Negative		1/2/50 generalized infiltration. Fine granular char.		3/27/52 sed. rate (1 hr.) 18 mm.
			3/26/52	-	-	-	-	2/ 6/52	Sputum	Mice	Negative				
										Culture	Negative				
			4/10/52	-	-	-	-	3/31/52	2 sputa	Culture	Negative				
										Mice		Negative			
			9/ 2/52	-	-	-	-	4/ 4/52	2 sputa	Culture	Negative	Negative	9/3/52 no change.	9/3/52 Shortness of breath but able to work.	9/3/52 sed. rate (1 hr.) 12 mm. 915 Roentgen units X-ray therapy R. chest 4/19/52.
										Mice	Negative				
								4/10/52	2 sputa	Culture	2 negative				
								4/11/52	2 sputa	Mice	2 negative				

*Skin tests: H, T, B, C refer to histoplasmin, tuberculin, blastomycin, and coccidoidin, respectively.
†H.c. refers to *Histoplasma capsulatum;* t.b. to tubercle bacillus.
‡L.3 refers to third interspace, left lung.

with vitamin capsules and cortisone, 50 mg. every 6 hours. The latter was decreased to 25 mg. every 6 hours and finally to 25 mg. 3 times a day. Cortisone ointment was also applied to the eyes. There was steady improvement in the patient's vision and decrease in the pulmonary symptoms and in the size of the lymph nodes. She was discharged, improved, after twenty-eight days in the hospital. There was slight clearing of the lung lesions as seen by x-ray. The biopsy sections of muscle and lymph node failed to reveal *H. capsulatum.* Following discharge from the hospital on Sept. 12, 1952, blurring of vision was again noted and examination revealed that new nodules had appeared in both eyes. Terramycin therapy, 2 Gm. daily, was carried on intermittently from Sept. 12, 1952, through Oct. 27, 1952. Her vision had shown marked improvement by November 1. About the first of September, small raised papular skin lesions were noted on the nose and cheeks. These had disappeared by November 1.

Comment: This case represents an unusual combination of sarcoidosis with ocular and lymph node lesions and histoplasmosis proved by culture. The patient has shown slow irregular improvement.

Laboratory Infection With Histoplasma With Moderately Severe Illness: Recovered.—

CASE 2.—The patient (P. A.), a white man, aged 28 years, a worker in the Histoplasmosis Laboratory, was a lifelong resident of Kansas City. He was studied because of an illness associated with a conversion of his histoplasmin skin test from negative to positive.

History: The patient developed a constant toothache in the left lower second bicuspid on Feb. 21, 1952. When this persisted for two days, he went to his dentist who found no cause for the pain. Three days later he developed a headache aggravated by movements of the head and, on the following day, generalized muscle pains and dull precardial chest pain. He also noted redness of the conjunctiva and some ocular fatigue as well as chilly sensations.

Admission findings: The patient was not admitted to a hospital. His temperature on March 4 was noted to be 99.6° F. A general physical examination was negative. White blood cell count was 8,900 with 62 per cent polymorphonuclear leukocytes, 32 per cent lymphocytes, 5 per cent eosinophils, and 1 per cent monocytes. Heterophile antibody titer was 1:64. Sedimentation rate was 17 mm. in 60 minutes. Chest x-rays were negative prior to onset of illness, but on March 4 a definite minimal infiltration of a pneumonic type was noted in the right fourth interspace and a rather diffuse pneumonic type infiltration in the left third interspace. The left hilar lymph nodes were enlarged.

Skin tests: The patient had 3 negative histoplasmin skin tests between the time of entering employment on February 4 and February 19 (Table I). On March 3, a large positive reaction was noted (induration 25 mm., erythema 55 mm.). The tuberculin test was positive; the blastomycin and coccidioidin tests, negative.

Mycology and bacteriology: Eight sputum specimens were negative for pathogenic fungi by culture and mouse inoculation.

Course: The pain in the tooth continued and irregular temperature elevation of around 100.5° F. persisted for approximately two weeks. A generalized weakness also persisted. Terramycin, 250 mg. three times a day, was begun on March 4 and gradually stepped up to 5.0 Gm. daily by March 9. It was discontinued on March 12. The patient continued to feel weak and have slight thoracic pain until March 24, when he was able to return to work after a three-week absence. He still had a slight toothache and chest pain. These symptoms persisted until about June 15. A weight loss of 16 pounds was noted. The sedimentation rate, which had fallen progressively to 8 mm. on March 24, rose slightly on April 10 and reached 19 mm. by April 16. Then it fell again slowly, reaching 8 mm. by July 17. Heterophile antibody titer on March 10 was 1:32. By March 18, x-ray showed that the lesion of the right lung had largely disappeared, although the one in the left appeared more discrete. By March 24 there was evidence of an increase in the pneumonic lesion in the left interspace. This increase was definite by April 16. Clearing of the lesion then began, and by September 30 the x-rays were largely negative except for a left hilar lymph node enlargement, which has since been gradually subsiding.

Summary: This case represents a moderately severe influenza-like illness with conversion of histoplasmin skin test, and positive serologic and x-ray changes.

Laboratory Infection With Histoplasma With Severe Illness: Recovered.—

CASE 3.—The patient (A. W.), a white woman, aged 49 years, a secretarial worker in the Histoplasmosis Laboratory, has lived in Kansas City for over twenty-two years. She was born in Nebraska. She was studied because of an illness accompanying a conversion in the histoplasmin skin test from negative to positive.

History: There was a gradual onset of malaise and headache on approximately the third of May, 1951. Subsequently, cough and slight chest pain developed, with daily temperature elevation of 100 to 101° F. After several days the chest pain disappeared but the headache, cough, weakness, and elevated temperature continued.

Admission findings: The patient was not admitted to a hospital. Physical examination by the family physician was consistently negative. The red cell count was 3.74 million with 75 per cent hemoglobin. The white cell count was 5,200, with polymorphonuclear leukocytes 68 per cent, lymphocytes 27, monocytes 4, and eosinophils 1 per cent. Urine was negative. Brucella agglutination test was also negative. The sedimentation rate was 25 mm. in 60 minutes. Chest x-rays were negative.

Skin tests: The patient had two negative histoplasmin skin tests during April before the development of her illness (Table I). Her histoplasmin skin test was definitely positive (12 mm. of induration and 32 mm. of erythema) approximately three weeks after the onset of her illness on May 21. This was five weeks after the last negative skin test. A tuberculin skin test was also positive.

Mycology and bacteriology: Due to the lack of sputum, no attempts at cultural identification of the etiological agent were performed.

Course: The temperature elevation of 100° to 101° F. continued with cough and weakness for about seven and a half weeks, then gradually subsided over the next week and a half, with the result that the patient was able to return to work after an absence of nine weeks. Sedimentation rate continued elevated until about October when it was still 12 mm. in 60 minutes. A definite pneumonic patch became apparent in x-rays of the chest on Nov. 21, 1951, about six and a half months after the onset of illness. This lesion was in the right third and fourth interspaces. It healed slowly and disappeared by May 15, 1952.

Summary: This patient may be considered to have been infected with *Histoplasma capsulatum* in the laboratory and subsequently showed x-ray, serologic, and skin test changes. The patient was ill for approximately nine weeks with influenza-like symptoms. No cultural studies were performed.

Laboratory Infection With Histoplasma With Moderately Severe Illness: Recovered.—

CASE 4.—The patient (F. B.), a white man, aged 64 years, had lived in Boston, Massachusetts, until he moved to the Kansas City area about two years before onset of illness. He was studied because of an illness accompanied by a change in his histoplasmin skin test from negative to positive.

History: This white janitorial worker in the Histoplasmosis Laboratory developed sore throat, chest pain, headache, and malaise on March 28. These symptoms continued for eight days, leaving the patient very tired and exhausted. On April 7, he had chills and fever with occasional cough and believed he had influenza. This illness continued for seven days, when the patient returned to work. The patient noted continued cough and sputum and some pain in the liver region together with a feeling of tiredness.

Admission findings: The patient was not admitted to a hospital. A physical examination was negative. Sedimentation rate was within normal limits. X-ray of the chest showed some pulmonary fibrosis which had been noted on previous x-rays.

Skin tests: This patient had 9 negative histoplasmin skin tests between April 10, 1951, and March 19, 1952 (Table I). On April 2, 1952, another histoplasmin skin test was given and read as negative at forty-eight hours. However, when the patient was examined on April 7, both skin test areas were red and swollen. A pigmented scaling area was still evident in

both test areas twenty days after the tests had been given. The test was repeated on April 22, and showed an area of induration of 15 mm. and an area of erythema of 50 mm. The tuberculin test was positive.

Mycology and bacteriology: Four sputum specimens were negative for fungi by culture and four were negative by mouse inoculation.

Course: The patient's illness continued for approximately one week before he returned to work. The cough, sputum, and pain in the liver region continued. Five months after onset the patient still had sputum and cough and complained of a constricted feeling in his chest and of fatigue. No weight loss was noted during his illness. No changes in x-ray were noted during the course of his illness.

Comment: This case is one of a laboratory infection with *Histoplasma* accompanied by fairly severe illness, skin test change, and serologic changes but no changes in the x-ray.

Probable Laboratory Infection With Histoplasma With Moderately Severe Illness: Recovered.—

CASE 5.—The patient (H. N.), a white man, aged 27 years, a physician, had been a lifelong resident of New York except for four years in Colorado. He was studied because of an illness and positive serologic tests for histoplasmosis (discussed in the following section).

History: The patient had been assigned to the Histoplasmosis Laboratory on Dec. 2, 1951. He did not remain on duty here, however, but went to Arkansas and visited a cave where a histoplasmosis epidemic had originated. This was on December 5. He continued on field assignments except for the last twelve days in December and the first twenty-one days in January. During this period of time he was daily in the Histoplasmosis Laboratory. Late in January the patient went to Colorado, where he became ill on February 29 with malaise, fatigue, and anorexia. The next day, March 1, his symptoms were more marked and accompanied by nausea and diarrhea. In the afternoon, chills and fever developed with a temperature of 103° F. Tightness in the chest and a mild nonproductive cough were also noted. On the following day, March 2, the temperature had subsided to 100° F., but the patient continued to feel weak and tired. These symptoms gradually abated over the course of the next week.

Admission findings: The patient was not admitted to a hospital. Physical examination was negative. The sedimentation rate was 6 millimeters. X-ray showed a definite minimal pneumonic type lesion in the right second interspace on March 31, 1952. A review of x-rays taken one year earlier showed this lesion had not then been present.

Skin tests: This patient was first skin tested on March 19, 1952, at which time the tuberculin, histoplasmin, and blastomycin skin tests were all positive, and coccidioidin was negative (Table I). This was twenty days after the onset of illness.

Mycology and bacteriology: No cultural studies were performed.

Course: The patient's symptoms of weakness and malaise continued for approximately a week after onset and he has had occasional malaise since that time. The x-ray lesion which was noted on March 20 progressively decreased although it was still evident in the film of Aug. 11, 1952. Sedimentation rate continued within normal limits until June 19, when a rate of 14 was noted. The rate was 15 on July 25, 20 on August 11, and had fallen to 8 by October 24.

Comment: The patient had lived in areas of low histoplasmin sensitivity all his life until he came to Kansas City. Although a negative histoplasmin skin test was not obtained, this patient probably was also infected in the laboratory with *Histoplasma* according to the positive serologic findings and positive skin tests.

Histoplasmosis With Sarcoidosis and Eye Lesions: Condition Improved.—

CASE 6.—The patient (U. C.), a white man, aged 50 years, a photographer, had been for thirty years a resident of a city 15 miles west of Kansas City. He was studied because of a positive serologic test for histoplasmosis.

History: Beginning in October, 1951, the patient noticed a deficiency of vision in the right eye. He consulted his family physician, who referred him to an eye specialist, who in turn referred him to the hospital. The patient stated that he had been found to have 20/20

vision six months before admission to the hospital. The patient had spent a considerable period of time working in the flood waters during the 1951 flood. He also had spent the past twelve summers in Northern Canada in the woods. The patient was notably prone to sties, of which he had had 6 in the past six months.

Admission findings: On Dec. 5, 1951, the positive findings on physical examination were a bilateral inguinal hernia, atrophy of the right arm, probably postpoliomyelitic, and retinitis of the right eye with a possible cyst of the retina of the left eye. The red blood count was 4.6 million, hemoglobin 91 per cent, white blood count 11,250, 44 per cent polymorphonuclear leukocytes, 52 per cent lymphocytes, 1 eosinophil, and 3 monocytes. The Wassermann test was negative, as was a urinalysis. The sedimentation rate was 14 mm. in one hour. Brucella agglutination test was negative. X-ray of the chest showed fibrosis in both apical areas, worse on the right with possible cavity formation.

Skin tests: On Dec. 5, 1951, blastomycin and tuberculin tests were negative, but histoplasmin and coccidioidin tests were positive (Table I). Repeat skin tests on Jan. 30, 1952, showed tuberculin, blastomycin, and coccidioidin tests negative, histoplasmin positive.

Mycology and bacteriology: Five gastric aspirations were done, of which two were positive for *H. capsulatum* by mouse inoculation. However, attempts to show *H. capsulatum* by cultures of these gastric washings were unsuccessful. Also, one sputum was negative for acid-fast bacilli by smear technique and 3 gastric washings were negative by culture at eight weeks.

Course: The patient's temperature remained normal throughout the period of hospitalization. After specimens were obtained for culture, he was discharged from the hospital on treatment of 25 mg. of Priscoline, taken three times a day. He was discharged after seven days in the hospital. He was seen on Sept. 3, 1952, at which time he had no complaints and was carrying on his usual work. His chest x-rays, however, showed what might have been a slight increase in infiltration in the first interspaces on both the left and the right.

Comment: This is another case of histoplasmosis and sarcoidosis with eye lesions. Diagnosis of histoplasmosis was made on the positive serologic findings, positive culture, and marked lung changes. He seems to be carrying on well and has shown no change in his clinical course.

Proved Histoplasmosis and Tuberculosis: Recovery Questionable.—

CASE 7.—The patient (P. C.), a white woman, aged 29 years, a housewife, had lived all of her life in Yates Center, Kansas, about 125 miles southwest of Kansas City, with the exception of two and one-half years in California and Oregon. She was studied because of positive serologic tests for histoplasmosis.

History: The patient had a five- or six-year history of productive cough and intermittent hemoptysis. During this period clubbing of the fingers gradually developed. She also experienced discomfort in the right chest. Approximately two years previously she had been hospitalized in a sanitarium in Oregon for six weeks, where she was told on discharge that she did not have tuberculosis. This was based on 9 negative sputum concentrates for tubercle bacilli. She had been hospitalized during this period because of abnormal findings in her chest x-ray.

Admission findings: On Nov. 1, 1951, the positive findings were diminished breath sounds at the right apex and clubbing of the fingers and toes. The liver and spleen were not palpated and the temperature was normal. The red blood count was 2.9 million, the hemoglobin 11 Gm., white blood count within normal limits. Total proteins were 8.5, globulin 2.98, albumin 5.53. X-rays showed a multiloculated cavity in the right upper lobe with some calcification in the left mid-lung. X-rays of the hands showed no abnormality.

Skin tests: Positive tuberculin and histoplasmin and negative blastomycin and coccidioidin tests were shown on two occasions.

Mycology and bacteriology: One of 6 sputum cultures was positive for *H. capsulatum,* while 2 of 5 sputa were positive by mouse inoculation. *H. capsulatum* was also isolated from the lung removed at operation and from the thoracic fluid. Tubercle bacilli were recovered from the draining chest fluid by culture and guinea pig inoculation. Eight sputum specimens obtained between the time of admission and the end of the year were negative by smear for

acid-fast bacilli, 4 of these were also negative by culture. In May, 1952, positive smears and cultures were obtained for tubercle bacilli on at least 6 occasions. The last specimen, obtained Oct. 24, 1952, was negative for tubercle bacilli by culture.

Course: On Dec. 5, 1951, a thick multiloculated cyst involving the right upper and middle lobes and the upper segment of the right lower lobe was resected. Postoperatively, the patient developed a pleural fistula requiring a resection of the remainder of the lobe and finally thoracoplasty. She was discharged on Dec. 22, 1951, but readmitted Jan. 7, 1952. On Feb. 5, 1952, the right lower lobe was removed and a rib thoracoplasty performed. She seemed to have fluid in the right chest and was readmitted on May 15, 1952. The complaint during this admission was pressure in the chest, cough productive of purulent material, and generalized malaise. During this admission positive cultures of acid-fast bacilli were found and the diagnosis of tuberculosis was confirmed by guinea pig inoculation. She was transferred to the tuberculosis hospital and has been under treatment with streptomycin and para-aminosalicylic acid. Her course to date has been satisfactory as indicated by decrease in sputum and increase in weight. She was in the hospital as of Nov. 2, 1952.

Comment: This is a case of histoplasmosis with marked lung changes resembling a cyst. Following the operation, complications developed and eventually the patient was found to have tuberculosis, a disease which was not detected during the first admissions. The ultimate prognosis is questionable.

Possible Histoplasmosis With Chronic Disease: Recovery Questionable.—

CASE 8.—The patient (R. H.), a white man, aged 44 years, a farmer, had spent his life in a small town in Kansas about 250 miles west and south of Kansas City. He was studied because of positive serum titers against *H. capsulatum* antigens.

History: The patient had been complaining of shortness of breath and cough for fifteen years. His illness began after he became chilled following exposure to dusty air. Shortly after this a cough developed which has persisted. Shortness of breath while at work first became apparent eight to ten years before admission. This had progressed until his activities were considerably limited by dyspnea and fatigue. Six years prior to admission a diagnosis of asthma was made. Two years before admission a diagnosis of moniliasis was made with subsequent treatment with iodides without improvement. Progressive cough, weakness, and dyspnea led to his hospitalization. There was an asthmatic family history.

Admission findings: On March 21, 1952, physical examination showed marked increase in the anteroposterior diameter of the chest with hyperresonance and fine crackling râles throughout. The accessory muscles were used in respiration. Red blood cell count was 5.14 million with 98 per cent hemoglobin. White cell count was 11,050 with 72 per cent polymorphonuclear leukocytes, 20 per cent lymphocytes, 4 per cent eosinophils, and 4 per cent monocytes. Urine was negative. Nonprotein nitrogen was 35. Total protein was 8.6 Gm., albumin 4.65, globulin 3.95. The sedimentation rate was 18 mm. in one hour. X-ray of the chest showed disseminated infiltrations throughout both lung fields, which were more marked about the hilar areas. There was also a suggestion of enlargement of the right heart. X-rays of the hands and feet were negative.

Skin tests: Skin tests with tuberculin, blastomycin, and coccidioidin were negative over the period between Feb. 4, 1952, and Sept. 2, 1952. Five tests with histoplasmin were negative during the same period.

Mycology and bacteriology: Three sputum specimens were negative for acid-fast bacilli by smear and culture. One gastric washing was negative for acid-fast bacilli by culture. Eight other sputum specimens were negative for pathogenic fungi by culture and mouse inoculations.

Course: Following a diagnosis of pulmonary fibrosis, x-ray therapy was tried on one side of the chest. A total of 915 roentgen units was given to both anterior and posterior right chest. He was dismissed without medication on April 12, 1952. He has shown some subjective improvement but his dyspnea is not markedly relieved. He has gained 5 pounds, however, and carries on his farm work. There has been no change in the chest x-ray during the period of observation.

Comment: Although the findings may be consistent with histoplasmosis, the organisms could not be found by cultural studies.

SEROLOGIC STUDIES

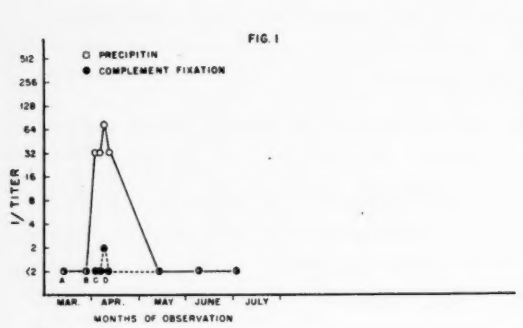
The 6 acute cases of histoplasmosis are somewhat similar to one another, both serologically and clinically.

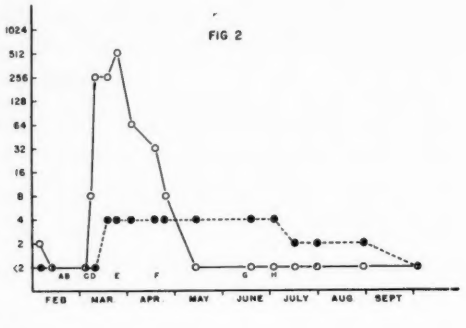
Patient 1 (G. W.), who had a relatively mild disease, showed humoral antibodies in her serum for a period of five weeks or less. Early in April, at the time the patient first displayed a skin sensitivity to histoplasmin, precipitins were observed (Fig. 1). Precipitins were barely demonstrable on March 26, but seven days later formed heavy precipitates in antigen diluted 1:32. These precipitins persisted for at least two weeks. When the serum was again examined in the middle of May, no precipitins could be detected. The fungus was isolated from gastric washings during the time when the titer in the precipitation test was at its peak. The complement-fixing antibody titers were negative, except for one doubtful titer of 1:2.

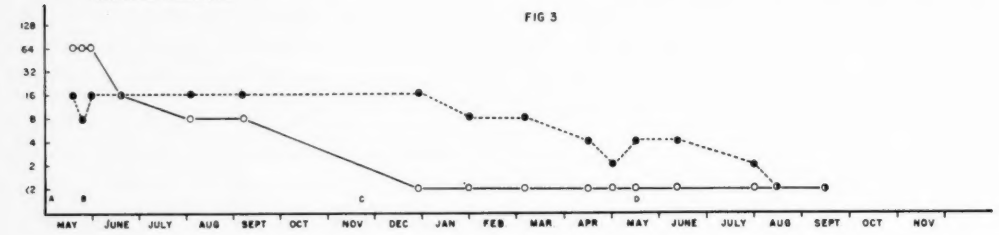
Patient 2 (P. A.) also showed a rapid and marked development of precipitins about two weeks after the first appearance of clinical symptoms and several days after a positive histoplasmin skin test (Fig. 2). This appearance of precipitins in the blood coincided also with a definite minimal infiltration in the lungs. Precipitins were first detected in early March, formed extremely heavy precipitates during the rest of March and April, and by May 14 were no longer measurable. The period immediately preceding the disappearance of precipitins coincided with the initiation of the clearing of the lesion in the left lung. During this period when precipitins were detectable, the complement-fixing antibodies remained at a constant level of 1:4, a titer which is so low that it is interpreted as indicating only a doubtful level of anti-*Histoplasma* antibodies. Although this case resembles that of Patient 1 very closely, and, in fact, seems more severe, no fungi were isolated from the sputum.

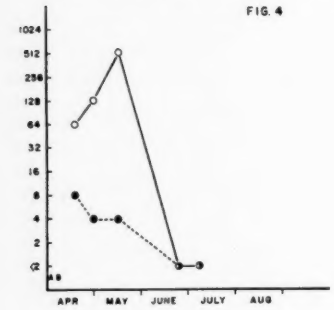
Patient 3 (A. W.) developed precipitins about one to two weeks after the onset of clinical disease (Fig. 3). These precipitins persisted from May to a date between September and December of the same year, a period of five to eight months. Complement-fixing antibodies were also present during mid-May, 1951, but persisted without loss of titer until the end of December. There was then a gradual decrease in titer until August, 1952 (fifteen months after onset), when no complement-fixing antibodies could be detected. The first signs of lung infiltration were not observed until November, at which time precipitation titers were almost negative. In May, 1952, about twelve months after onset, when lung lesions were no longer apparent, precipitation titers had been nondetectable five months, while complement-fixation titers were at a doubtful level of 1:4.

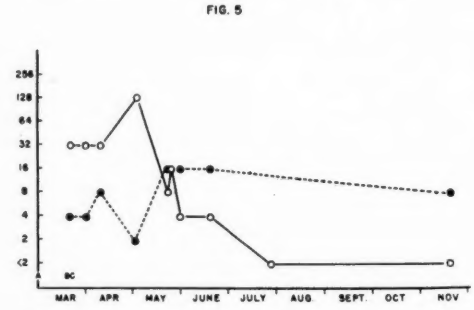
Patient 4 (F. B.) had precipitins from the time of the first test in mid-April until May 16, a period beginning two to three weeks after onset of clinical symptoms and less than two weeks after the first appearance of a positive histoplasmin test (Fig. 4). Complement-fixing antibodies were also detected

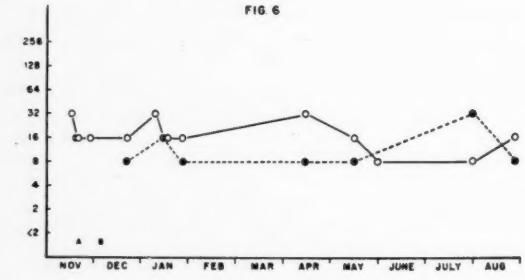


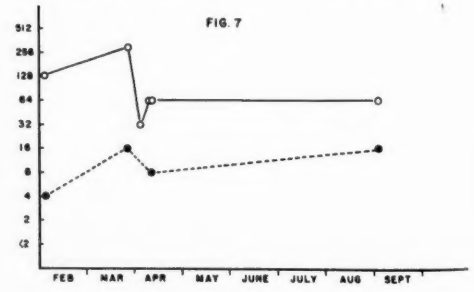












Figs. 1-7.—Serologic results from complement-fixation and precipitin tests, with indications of important clinical and laboratory findings.

Fig. 1.—Patient 1 (G. W.): *A*, Negative skin test with histoplasmin. *B*, Positive histoplasmin skin test. *C*, Lymph node and bone marrow negative by culture. *D*, Gastric washings positive for *H. capsulatum* by mouse inoculation.

Fig. 2.—Patient 2 (P. A.): *A*, Negative histoplasmin skin test. *B*. Onset of clinical symptoms. *C*, First positive histoplasmin skin test; patient left work. *D*, First pneumonic infiltration in right lung. *E*, Patient resumed work; increase in lesion in left interspace detected. *F*, Clearing of lung lesions. *G*, Complete disappearance of clinical symptoms. *H*, X-rays largely negative.

Fig. 3.—Patient 3 (A. W.): *A*, Onset of malaise and headache. *B*, Positive histoplasmin skin test. *C*, First positive x-ray. *D*, Lung lesion no longer apparent.

Fig. 4.—Patient 4 (F. B.): *A*, Onset of symptoms. *B*, Skin test negative to histoplasmin. *C*, Skin test positive to histoplasmin.

(Legends continued on opposite page.)

in low titers during this period. When the patient's serum was again tested about one month later, neither precipitins nor complement-fixing antibodies could be detected.

Patient 5 (H. N.) had precipitins when he was first studied about three weeks after the initial appearance of clinical symptoms (Fig. 5). At this time, a pneumonic lesion was observed by x-ray examination. The precipitins were detectable for a period of about five months. Complement-fixing antibodies gradually rose to a titer of 1:16 during the fourth month after onset, and were still present to a titer of 1:8 in November, about nine months after onset.

Patient 6 (U. C.) showed distinct antibodies, both complement-fixing and precipitating, on only one date. Thereafter, precipitation titers were either low or negative (Table II), and complement fixation, negative. Nevertheless, repeated attempts to demonstrate an etiologic agent were finally positive when *H. capsulatum* was isolated from gastric washings.

TABLE II. SEROLOGIC RESULTS ON PATIENT 6 (U. C.) WITH WHOLE YEAST CELLS AS THE COMPLEMENT-FIXATION ANTIGEN AND MYCELIAL FILTRATE AS THE PRECIPITATING ANTIGEN

	TITERS	
	COMPLEMENT-FIXATION	PRECIPITIN
Dec. 19, 1951	32	64
Jan. 30, 1952	0	2
Feb. 1, 1952	0	4
March 10, 1952	0	4
Sept. 2, 1952	0	0

The 2 patients with chronic histoplasmosis showed a different antibody response from that of the 6 previous patients.

Patient 7 (P. C.) had a more severe clinical disease and a more persistent antibody response (Fig. 6). When her serum was first assayed on Nov. 16, 1951, precipitins were detectable in an antigen dilution of 1:32. These antibodies were present throughout the period of study (ten months), together with the complement-fixing antibodies, which remained at almost a constant level.

Patient 8 (R. H.) also showed the presence of precipitins and complement-fixing antibodies during the almost eight months of study (Fig. 7). The complement-fixing antibodies were detected at a 1:4 serum dilution when the patient was first studied, and had risen to a titer of 1:16 seven months later.

The sera from the foregoing 8 patients were also tested for precipitins in the presence of the heterologous antigens, blastomycin (from *Blastomyces dermatitidis*) and coccidioidin (from *Coccidioides immitis*). Cross reactions were manifest in the sera from all 8 patients, especially with sera which had high titers with the homologous antigen. Although the heterologous antigens

(Continued from opposite page.)

Fig. 5.—Patient 5 (H. N.): *A*, Onset of clinical symptoms. *B*, Skin test positive with histoplasmin. *C*, Pneumonic lesion observed.

Fig. 6.—Patient 7 (P. C.): *A*, Sputum positive for *H. capsulatum* by culture and by mouse inoculation. *B*, *H. capsulatum* isolated from lung removed at operation.

Fig. 7.—Patient 8 (R. H.).

[Note: Titer refers to the antigen-dilution in the precipitation test, and to the serum-dilution in the complement-fixation test.]

were tested against at least 1 or 2 serum samples from each patient, a series was obtained only from Patients 2, 4, and 5. Precipitates with the homologous antigen extended over a longer period of time, and usually were present at a higher antigen dilution than with the heterologous antigen.

It may be added at this point that the precipitation is believed not to be just an acute-phase reaction. Tests with human sera from the acute stages of 3 cases of brucellosis, 3 of tularemia, 2 of Q fever, 2 of spotted fever, 1 of Shigella dysentery, 3 of nasopharyngitis, 2 of infectious mononucleosis, 2 of acute follicular tonsillitis, 2 of primary atypical pneumonia, 2 of "summer grippe," and 1 of Vincent's angina showed no precipitates in antigen dilutions of 1:2 and higher. Similarly, of 524 sera from histoplasmin-negative patients of the University of Kansas Medical Center, 502, or 96 per cent, showed no detectable precipitins.

DISCUSSION

Surveys based on skin tests performed with histoplasmin as antigen have indicated the probable existence of large numbers of mild or acute cases of histoplasmosis in which the infections remain localized and are nonfatal. The detection of mild active cases has been relatively rare, because of the difficulty in isolating the etiological agent and because of the absence of a suitable diagnostic aid. The complement-fixation test has been helpful in indicating chronic cases and to a much more limited extent in discovering acute cases. Many patients, however, who were mildly ill and who changed from a recent histoplasmin-negative to histoplasmin-positive state showed no marked changes in complement-fixing antibodies.

The precipitin test, therefore, appears to be a useful tool in the diagnosis of acute cases of histoplasmosis. On the basis of serologic and clinical findings, 6 of the foregoing 8 patients may be considered to have had acute infections. In these 6, precipitins were detected for periods from one to six months, although in 3 of the patients the antibodies were apparent for only one to two months. The time interval between a negative and a maximum titer sometimes was only a few days. In 2 of the patients, only doubtful complement-fixing antibodies were detected; in 3 others, the complement-fixing antibodies were low, not exceeding a 1:16 titer; and in one, the complement-fixation titer reached 1:32. In Patient 3 (A. W.) the complement-fixing antibodies persisted in low titers for over a year. Patient 1 (G. W.), from whom *H. capsulatum* was isolated, showed no complement-fixing antibodies, with the exception of one bleeding with a 1:2 titer. Her precipitins lasted for only one month. It is to be emphasized that 5 of 6 patients with acute infection converted to a histoplasmin-positive skin sensitivity shortly before the appearance of precipitins. The sixth, Patient 6, was positive when first studied. Also, all 6 patients are recovering or have apparently recovered clinically. Another point illustrated especially by Patients 1 and 6 is the necessity for frequent serologic tests, so that a transitory but diagnostic rise in antibody titer may not be missed.

Patient 7 (P. C.) and Patient 8 (R. H.), who showed signs of chronic histoplasmosis, had complement-fixing antibodies and precipitins that were evident with little change over the periods of study, namely, nine and twelve months. In both these cases, higher titers were obtained in the precipitation test than in the complement-fixation test during the early phases of study. Cross reactions were manifest, but titers with the heterologous antigens were lower or of shorter duration.

The precipitation is believed not to be an anamnestic response brought about by skin-testing previously sensitized persons with histoplasmin. In a group of 32 students skin tested for five consecutive weeks, not one showed a marked rise in precipitation titers.[14]

The concurrent determination of the precipitins and complement-fixing antibodies may furnish valuable prognostic as well as diagnostic aid, as in coccidioidomycosis.[12] In Smith's experience, the persistence of antibodies reacting in both precipitin and complement-fixation tests over a period of time suggests generalization of the infection. It is interesting to note that both Patient 7, the proved case who is doing poorly, and Patient 8, the suspected case who is not improved, show this phenomenon. Both complement-fixing antibodies and precipitins in Patient 7 have persisted unchanged at relatively high levels for at least eleven months. In Patient 8, similar findings have occurred over a period of eight months.

In coccidioidomycosis, Smith and associates[12] found that the absence of complement fixation was generally associated with a milder infection. While nearly every patient with coccidioidomycosis whose serum fixed complement would, if his serum had been tested early enough, have demonstrable precipitins, many patients whose serum had precipitins never fixed complement.

The serologic response of patients with histoplasmosis may parallel that of patients with coccidioidomycosis. The two mildest cases (Patients 2 and 4) had demonstrable precipitins for about two months each, with complement-fixing titers either of doubtful significance (Patient 2) or of very short duration (Patient 4). In Patient 5, who had a more severe clinical disease, a precipitin titer lasted three to four months while the complement-fixing antibodies remained for seven months. Finally, in Patient 3, who showed the most severe clinical course, the precipitins remained elevated for at least five months and the complement-fixing antibodies for fourteen months before dropping to the normal range. That this parallelism does not invariably hold, however, is shown in Patient 1, who showed only a transient precipitin rise and no complement-fixation changes although her sputum was positive by culture for the fungus and she is not completely recovered. Two significant factors may explain the findings in her case. First, she is a Negro, and, second, her condition has been diagnosed as having been complicated by sarcoidosis. It would appear from the data, therefore, that the combined determination of repeated precipitin and complement-fixing antibody titers may furnish valuable prognostic aid in the individual case.

SUMMARY

Precipitin tests, with the filtrate of broth cultures of the mycelial phase as antigen, and complement-fixation tests, with the whole cells of the yeastlike phase of *Histoplasma capsulatum* as antigen, were used to study the humoral antibody response of 8 patients. Three of the patients with a brief mild disease showed no marked complement-fixing antibodies, but had precipitins which were detected over a period of two to three months. Three of the patients with a more severe acute disease exhibited for a relatively brief but variable period both complement-fixing antibodies and precipitins, with the former observable for a longer time. Two other patients who were chronically ill had precipitins and complement-fixing antibodies persisting for months without a marked decline. These changes in antibody titers were correlated with the clinical condition of the patient, the x-ray picture, and the laboratory findings.

Addendum.—Since these experiments were performed, four additional culturally proved cases of histoplasmosis have been found in which the sera showed marked precipitins but only low-titered complement-fixing antibodies.

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CONCENTRATION OF ANTIBIOTICS IN THE BRAIN

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NEO-PENIL† (the diethylaminoethyl ester hydriodide salt of penicillin G) is a relatively new form of penicillin for parenteral use. It has been suggested that for some reason, as yet unexplained, this substance, as well as other penicillin esters, has an affinity for certain tissues. Several studies have been reported which indicate that Neo-Penil is concentrated in the pulmonary tissue. Jensen and hs colleagues[1] reported significant concentration of Leocillin (benzylpenicillin-β-diethylaminoethylester hydriodide) in brain tissue of guinea pigs. Schimmel and his co-workers[2] found that Neo-Penil produced cerebrospinal fluid levels in the human about ten times greater than the levels produced by similar doses of other types of penicillin. Flippin and his associates[3] demonstrated that Neo-Penil produces lower and less prolonged plasma concentrations than those resulting from procaine penicillin. The following study was undertaken in an attempt to determine the concentrations of Neo-Penil, as well as several other antibiotic agents, in the human brain. In a review of the literature we were unable to find reference to any study concerning the concentrations of antibiotics in living human brain tissue. Baggenstoss and his colleagues[4] reported that no streptomycin was found in the brain tissue at necropsy. It is hoped that the method of investigation outlined in this study will result in further studies concerning the concentrations of various drugs in the human brain.

MATERIALS AND METHODS

The patients studied in this investigation had been hospitalized because of psychiatric disorders at the Rochester State Hospital. There was no evidence of infectious disease involving the brain or meninges in any of these patients. For this reason it seemed safe to conclude that specimens removed from the brain at operation would represent normal brain tissue. These patients were to undergo prefrontal lobotomy for therapeutic reasons. This operation was carried out in the usual manner following the technique described by Lyerly and modified by Love[5] for use at the Mayo Clinic and at the Rochester State Hospital. Following prefrontal lobotomy from the superior approach a specimen of brain tissue was removed in each case from the tract left by the Killian nasal septum elevator used for performing the prefrontal lobotomy. In each case it was attempted to remove somewhat more

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TABLE I. CONCENTRATIONS OF ANTIBIOTICS IN THE SERUM, CEREBROSPINAL FLUID AND BRAIN

PATIENT	ANTIBIOTIC	DOSE	METHOD OF ADMINISTRATION	AMOUNT IN SERUM, PER ML.	AMOUNT IN CEREBROSPINAL FLUID, PER ML.			AMOUNT IN BRAIN TISSUE, PER GM.	LEAST AMOUNT DETECTABLE IN BRAIN TISSUE PER GM.
					LUMBAR	CISTERNAL	LEAST AMOUNT DETECTABLE		
1	Neo-Penil	300,000 units, 24 hr. and 4 hr. preoperatively	I.M.	0.5 unit		0.06 unit		0.09 unit	
2	Neo-Penil	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.		0.06 unit	0.12 unit		0.18 unit	
3	Neo-Penil	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	2.0 units	0.06 unit	0.12 unit		0.36 unit	
4	Neo-Penil	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	0.5 unit	0.03 unit	0.03 unit		0.25 unit	
5	Neo-Penil	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	2 units	0.12 unit	0.12 unit		0.75 unit	
6	Neo-Penil	500,000 units, 28 hr. and 500,000 units 4 hr. preoperatively	I.M.	1.0 unit	0.06 unit	0.12 unit		0.48 unit	
7	Procaine penicillin-G	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	1.0 unit	None	None	0.03 unit	None	0.09 unit
8	Procaine penicillin-G	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	2.0 units	None	None	0.06 unit	None	0.18 unit
9	Procaine penicillin-G	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	1.0 unit	None	None	0.03 unit	None	0.12 unit
10	Procaine penicillin-G	600,000 units, 24 hr. and 4 hr. preoperatively	I.M.	0.06 unit	None	None	0.03 unit	None	0.24 unit
11	Erythromycin	300 mg. every 6 hr. for 24 hr. preoperatively	Oral	2.0 μg.	None	0.125 μg.	0.125 μg.	0.375 μg.	
12	Erythromycin	300 mg. every 6 hr. for 24 hr. preoperatively	Oral	8.0 μg.	0.125 μg.	0.125 μg.		0.375 μg.	

13	Erythromycin	500 mg. every 6 hr. for 24 hr. preoperatively	Oral	4.0 μg.	None	0.125 μg.	0.125 μg.	None	0.5 μg.
14	Erythromycin	500 mg. every 6 hr. for 24 hr. preoperatively	Oral	0.125 μg.	None	None	0.125 μg.	None	0.5 μg.
15	Erythromycin	500 mg. every 6 hr. for 24 hr. preoperatively	Oral	4.0 μg.	0.125 μg.	0.25 μg.		None	0.5 μg.
16	Dihydrostreptomycin	1 Gm., 24 hr., 12 hr., and 4 hr. preoperatively	I.M.	16.0 μg.	0.25 μg.	None	0.25 μg.	None	1.5 μg.
17	Dihydrostreptomycin	1 Gm., 24 hr., 12 hr., and 4 hr. preoperatively	I.M.	16.0 μg.	0.25 μg.	0.25 μg.		None	1.5 μg.
18	Dihydrostreptomycin	1 Gm., 24 hr., 12 hr., and 4 hr. preoperatively	I.M.	32.0 μg.	1.0 μg.	0.5 μg.		None	0.75 μg.
19	Dihydrostreptomycin	1 Gm., 24 hr., 12 hr., and 4 hr. preoperatively	I.M.	32.0 μg.	1.0 μg.	0.5 μg.		None	0.75 μg.
20	Terramycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	16.0 μg.	None	None	0.25 μg.	1.5 μg.	
21	Terramycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	8.0 μg.	0.25 μg.	None	0.25 μg.	None	1.25 μg.
22	Terramycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	8.0 μg.	0.125 μg.	0.125 μg.		0.375 μg.	
23	Terramycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	4.0 μg.	Trace	0.125 μg.	0.125 μg.	0.375 μg.	
24	Aureomycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	2.0 μg.	None	None	0.06 μg.	0.1875 μg.	
25	Aureomycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	4.0 μg.	None	0.0312 μg.	0.03 μg.	0.1875 μg.	
26	Aureomycin	750 mg. every 6 hr. for 24 hr. preoperatively	Oral	4.0 μg.	None	None	0.06 μg.	0.5 μg.	
27	Aureomycin	500 mg., 12 hr. and 4 hr. preoperatively	I.V.	16.0 μg.	None	None	0.06 μg.	0.5 μg.	

than 1 gram of brain tissue. There were no postoperative complications except for the loss of one bone button following operation. Prior to the time of operation these patients received for prophylaxis one of the following drugs: Neo-Penil, procaine penicillin-G in aqueous suspension, aureomycin, Terramycin, dihydrostreptomycin or erythromycin. Neo-Penil was administered by intramuscular injection in amounts of 300,000, 500,000 or 600,000 units 24 hours and 4 hours before operation. In a similar manner 600,000 units of procaine penicillin-G in aqueous suspension was given 24 hours and 4 hours preoperatively. Terramycin and aureomycin were administered orally in doses of 750 mg. every 6 hours for 24 hours before operation. In 1 patient oral administration was impossible and this patient received 500 mg. of aureomycin intravenously 12 hours and 4 hours prior to operation. Erythromycin was administered orally in doses of 300 or 500 mg. every 6 hours for 24 hours before operation. One gram of dihydrostreptomycin was administered intramuscularly 24, 12 and 4 hours prior to operation. At the time of operation, blood, cisternal fluid, cerebrospinal fluid from the lumbar area, and brain tissue were collected for antibiotic assay. Brain tissue for assay was weighed and thoroughly emulsified in a mortar together with an appropriate amount of isotonic saline solution. The methods used in the assay of these substances were as follows: Specimens from patients receiving Neo-Penil and procaine penicillin were assayed for penicillin activity using the Fleming slide cell with a hemolytic Streptococcus as the test organisms.[6] The standard titrations indicated that the test was able to detect as little as 0.03 to 0.06 unit of penicillin per milliliter. Assays for erythromycin were carried out by the same method. The procedure regularly detected down to 0.125 μg per milliliter. Concentrations of aureomycin and Terramycin were determined by means of a tube dilution method with thioglycollate broth and using a strain of *Bacillus cereus* as the test organism.[7] From 0.015 to 0.03 μg. per milliliter of aureomycin and from 0.06 to 0.12 μg per milliliter of Terramycin could be detected in this way. The assays for dihydrostreptomycin were performed by a tube dilution technique using nutrient broth and a strain of Klebsiella pneumoniae as the test organism. The limit of sensitivity with the test was 0.12 microgram per milliliter.

RESULTS

A total of 27 patients received one of the antibiotics used in this study (Table I). Each antibiotic agent was administered to 4 patients with the exception of erythromycin which was given to 5 patients and Neo-Penil which was given to 6 patients. When there was no antibiotic activity the least amount detectable as calculated from the end point of the standard titration and the dilution of the specimen has also been reported.

Of the 6 patients who received Neo-Penil it will be noted that in 1 it was impossible to obtain blood for assay and in another cerebrospinal fluid from the lumbar space could not be obtained. However, it is apparent that following the intramuscular administration of Neo-Penil, significant concentrations of this drug occurred in the serum, cerebrospinal fluid, and brain tissue. On the other hand, assay after the intramuscular administration of procaine penicillin-G in aqueous suspension revealed therapeutic concentrations of this antibiotic in the serum, but none was detectable in the cerebrospinal fluid or the brain tissue. Dihydrostreptomycin was not found in the brain but significant amounts were reported in the serum and cerebrospinal fluid. Aureomycin, like Neo-Penil, was found consistently in the serum and brain tissue. Although it has been established that aureomycin crosses the blood-brain barrier into the cerebrospinal fluid, none was found in this study. This may be explained by the fact that aureomycin is unstable and the cerebrospinal fluid was not re-

frigerated immediately. Adequate concentrations of erythromycin were demonstrated in the serum but only small amounts or none were found in the cerebrospinal fluid or brain. Terramycin differs from erythromycin in that in 3 instances significant amounts were demonstrated in the brain.

SUMMARY

Twenty-seven patients hospitalized for psychiatric disorders were given prophylactically one of the following antibiotics: Neo-Penil, procaine penicillin G in aqueous suspension, dihydrostreptomycin, erythromycin, aureomycin or Terramycin. These drugs were administered prior to prefrontal lobotomy. At the time of operation specimens of blood, cerebrospinal fluid and brain tissue were collected for assay.

The data presented in this study indicate that following the administration of several antibiotic agents only Neo-Penil and aureomycin can be detected consistently in significant amounts in the brain tissue. However, we would also like to call attention to the fact that Terramycin was present in the brain tissue of 3 of 4 patients. These findings may be of some significance in connection with the use of various antibiotic agents in the treatment of infections of the central nervous system.

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A SIMPLIFIED METHOD FOR TESTING SULFONAMIDE RESISTANCE OF GROUP A STREPTOCOCCI

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INTRODUCTION

THE development of satisfactory methods for measuring sulfonamide resistance of bacteria in vitro has been difficult because some of the materials used in bacteriologic media, particularly meat infusions and peptones, contain substances that antagonize sulfonamide action. In the case of microorganisms with simple growth requirements, such as *E. coli,* a synthetic medium composed of inorganic salts, dextrose, and asparagin, for example, can be employed, and is free from sulfonamide antagonists.[1] The use of a fully synthetic medium for fastidious organisms such as the hemolytic streptococci is not possible, because there does not exist a medium which will promote good growth of all streptococcal strains from a small inoculum. In 1945 the author presented a method for testing the sulfonamide resistance of hemolytic streptococci,[2] in which the semisynthetic medium of Adams and Roe[3] enriched with horse serum was used. This method was quite satisfactory, and extensive experience with it in connection with sulfadiazine prophylactic programs in the Armed Services demonstrated its ability to differentiate between strains which varied in sulfonamide resistance to an epidemiologically significant degree.[4] The method had one severe drawback. It was exceedingly laborious and time consuming, since its preparation involved the weighing of small amounts of vitamins and growth factors, sterilization of solutions by bacterial filtration, and combination of many solutions by pipetting with sterile technique. For this reason, the method has not been widely adopted and several alternative methods which are much simpler to perform have been proposed. Some of these use peptones which contain large amounts of sulfonamide antagonists and are for that reason unsatisfactory. Others use whole blood, which is not a suitable medium for the growth of steptococci, particularly because of the development of alkalinity from the diffusion of CO_2.

The purpose of this paper is to present a simplified and improved method for determining sulfonamide resistance of group A streptococci, in which the advantages of the semisynthetic medium base have been retained, but in which a large part of the labor has been eliminated by means of a dehydrated base.*

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*Available from the Difco Laboratories, Detroit, Mich.

MATERIALS AND METHODS

The basal medium, the composition of which is given in Table I, is in powdered form and merely has to be hydrated with distilled water, sterilized by autoclaving, and enriched with normal horse serum. Sodium sulfadiazine (S.S.D.) solutions are added to give the desired range of concentrations. The medium is semisolid, as in the earlier test, so that the inoculated streptococci grow as colonies in suspension and the size of the inoculum given can be estimated. The content of sulfonamide-antagonists is very low, as is shown by the inhibition of growth of many group A streptococci by as little as 0.1 mg. sodium sulfadiazine per 100 ml. of final medium.

Preparation of SR Medium.—Add 5.4 Gm. SR Medium Base (Difco) to 300 ml. distilled water. Boil 5 minutes to dissolve and restore to original volume with distilled water. Distribute 89.0 ml. to each of 3 flasks and autoclave at 15 pounds pressure for 15 minutes. (A whitish flocculent precipitate forms on autoclaving but will not interfere with test.) The pH is approximately 7.4 and does not need adjusting.

TABLE I. COMPOSITION OF SR MEDIUM BASE

	GM.
Casamino Acids Vitamin Free (Difco)	100.000
Dextrose	15.000
Sodium acetate	10.000
l-cystine	1.000
dl-tryptophane	1.000
Adenine sulfate	0.100
Xanthine	0.100
Uracil	0.100
Thiamine HCl	0.010
Riboflavin	0.006
Niacin	0.010
Calcium pantothenate	0.020
Pyridoxine HCl	0.020
Biotin	0.000004
Dipotassium phosphate	10.000
Disodium phosphate	30.000
Magnesium sulfate	1.000
Ferrous sulfate	0.100
Manganous sulfate	0.100
Agar	10.000

TABLE II. DILUTION SCHEDULE FOR SCREENING TEST. SR TEST MEDIUM FOR 48 STRAINS

	FLASK 1	FLASK 2	FLASK 3
CONCENTRATION OF SODIUM SULFADIAZINE IN MG. PER 100 ML.	0	5	25
SR Medium Base	89.0	89.0	89.0
Sodium Sulfadiazine 0.5%	-	1.0	-
2.5%	-	-	1.0
0.85% NaCl solution, sterile	1.0	-	-
Normal horse serum, sterile	10.0	10.0	10.0

Performance of Test.—Pure culture of the strains to be tested is inoculated into 5 ml. of blood broth and incubated overnight at 37° C. (Heart Infusion Broth [Difco] to which 1 drop of sterile sheep, rabbit, or horse blood is added per 5 ml. is satisfactory.) The flasks of SR Medium Base are removed from autoclave and placed in a water bath at 45° C., where they are allowed to stand a few moments for temperature equilibration. Saline solution, sodium sulfadiazine, and normal horse serum are added to the flasks according to the dilution schedule in Table II and mixed thoroughly. For each strain to be tested 4 sterile 13 by 100 mm. tubes are set up. To the first add 2 ml. of Barbital-Ringer's Gelatin (BRG) solution. To the remaining 3 add 2 ml. from flasks 1, 2, and 3, respec-

tively. Transfer a small loopful of the overnight blood broth culture of the strain to be tested to the tube containing BRG solution. Agitate this tube sharply to mix, then transfer a loopful from it to each of the three tubes containing SR Test Medium, and agitate the three tubes sharply to distribute the inoculum evenly. Incubate at 37° C. Inspect for visible growth after 24 hours' incubation. Discard the tube containing BRG.

Interpretation.—Strains which grow only in the first tube are sulfonamide sensitive; those growing in the first two tubes are moderately sulfonamide resistant; those growing in all three tubes are highly sulfonamide resistant.

Sodium Sulfadiazine Solutions.—Solutions containing 0.5 and 2.5 Gm. of sodium sulfadiazine in 100 ml. of N/100 NaOH are made with quantitative precision. In our experience it has not been necessary to sterilize these solutions. The solutions may be immersed in a boiling water bath for 10 minutes if desired. They will keep indefinitely without crystallization if placed in a stoppered vessel.

Barbital-Ringer's-Gelatin (BRG) Solution.—The composition of this material is given in Table III. The solution may be compounded as indicated there, or the standardized reagent may be obtained from Difco Laboratories, in which case 1.10 Gm. of the powder is dissolved in 100 ml. distilled water and sterilized at 15 pounds pressure for 15 minutes. The pH should be 7.3 to 7.4.

TABLE III. BARBITAL-RINGER'S GELATIN (BRG) DILUTING SOLUTION

Barbital (Diethyl barbituric acid)	0.575
Sodium Barbital	0.375
NaCl	8.50
KCl	0.25
$CaCl_2$	0.30
Gelatin	1.00
Distilled water to	1,000.00

Comments on Performance of Test.—In the control tube there should appear ten to several hundred colonies. If fewer than ten grow out, the test should be repeated using a somewhat larger inoculum. If the inoculum is too heavy, but growth is still inhibited in both tubes containing S.S.D., the test need not be repeated, since the strain is assuredly sulfonamide sensitive. The test may be modified to employ more tubes and other concentrations of sulfonamide. Many streptococcal strains are susceptible to 0.1 and 0.2 mg. per cent. A suitable inoculating loop is a platinum-rhodium loop with fused joint for delivering 0.001 ml. sample.* Or a loop may be made by winding 26 gauge platinum wire around an 18 guage intravenous needle. Horse serum must be used in the test. Rabbit serum used in this medium prevents sulfonamide from inhibiting a few nonresistant strains. The Barbital-Ringer's Gelatin solution is a nonnutrient, relatively nontoxic fluid in which group A streptococci will neither multiply nor die off in the course of several hours at room temperature. If desired, sterile 0.85 per cent NaCl solution may be used instead, provided the test is set up rapidly and the streptococci do not remain diluted in the saline more than a few moments before they are transferred to the final tubes of the test. The finished SR Test Medium may be dispensed into tubes the day before use, and kept at room temperature overnight. Evaporation and consequent concentration of sodium sulfadiazine is negligible. Although the medium appears quite viscous, the inoculum can be distributed evenly throughout it, and preliminary warming is not necessary. Preliminary experience has indicated that some batches of horse serum are not suitable for the test. If good growth is not obtained in the medium with the serum employed, another lot of serum should be tried. Lots of Difco dehydrated and liquid tissue culture horse serum have been found to be highly satisfactory in the test. The dehydrated serum has the advantage of better storage capacity and hence availability, and should be particularly useful in laboratories that need to perform only small numbers of tests occasionally.

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RESULTS

Range of Resistance.—One hundred and sixty-nine stock strains of group A streptococci, which had been preserved in the dried state, and which had originally come from numerous sources, representing all the known serologic types, were tested for sulfonamide resistance. In no case were the strains able to grow in 5 mg. or more of sodium sulfadiazine per 100 ml. of medium.

A distinct class of strains, not included above, consisted of those that, on epidemiologic or clinical grounds, were known to be resistant to sulfonamide action. One of these, C440, was a nonhemolytic group A, Type 12 strain originally isolated by Francis[5] from a ward epidemic in Britain. It grew in 25 mg. sodium sulfadiazine per 100 ml. of medium. Other strains belonged to Types 17, 19, and 3, and were isolated from epidemics in recruit camps in the U. S. Navy during World War II.[4] These strains were isolated during the course of sulfadiazine prophylaxis programs at a time when the prophylaxis had become ineffective because of drug resistance of the strains. They were able to grow in the presence of 5 or 25 mg. per cent sodium sulfadiazine (S.S.D.).

It is thus apparent that in this test strains, which from their origin gave no reason to be suspected of sulfonamide resistance, were unable to grow in 5 mg. per cent S.S.D. or more; whereas strains that were suspected of being resistant to sulfonamides on clinical or epidemiologic grounds were able to grow in the presence of 5 or 25 mg. per cent S.S.D.

Using the dehydrated base described in this paper and a selected batch of fresh horse serum, Gezon and his collaborators[6] tested 1,618 strains of group A streptococcus during an epidemic at the Bainbridge Naval Training Center in 1952. Of these only 3 strains showed evidence of drug resistance in the screening test. All others were sensitive to 5 mg. per cent or less. Two of the 3 resistant strains came from the same patient; the first was obtained before chemoprophylaxis with sulfadiazine was started and the second while the recruit was receiving sulfadiazine. These strains have not been identified serologically, but appear to be related to Type 14, and may be a new serologic type. The third strain belonged to Type 14 and was isolated from a recruit receiving penicillin prophylaxis. The origin of the resistance of these strains is not clear. It is highly unlikely that they were produced during the sulfonamide prophylaxis program, in which the drug was given only two weeks, particularly since one of the strains was isolated before the program was started and another appeared in a man not receiving sulfonamide. Type 14 strains resistant to sulfonamide were not encountered in extensive testing among military populations during World War II.

DISCUSSION

The method presented herewith represents a great simplification in time and labor over the earlier method of which it is a modification. Although it is not as simple as testing antibiotic sensitivity by filter-paper disk methods,

it compares favorably with the test-tube methods that are used for such testing.

The necessity for using horse serum as a nutritional supplement is regrettable, but until a fully adequate, fully defined medium for group A streptococci is devised, some sort of supplementation is required. Horse serum must be used in the test. Rabbit serum, for reasons which are unknown, allows a few strains of sulfonamide-sensitive streptococci to grow (although poorly) in high concentration of S.S.D.

The chief difficulty experienced in the performance of the test has been a deficiency in some horse sera used as supplements. Attempts to determine the nature of the difference among horse sera have not been successful, beyond showing that it is due to a lack of some growth-promoting principle and not to a growth-inhibitory effect. With an acceptable batch of serum, growth in the control tube should be good, by which is meant that the individual colonies, unless too crowded, should be well developed in size and recognizable as such without any difficulty. There is considerable difference in the colonial form taken by various streptococcal strains in the semisolid medium. Some of them grow in large, spherical, relatively diffuse balls, whereas others grow in a dense, compact form. Still others will form a small dense nidus from which a streamer of less dense growth extends downward. In any case there should be little difficulty in recognizing adequacy of growth, and no compromise can be allowed on that score. If a particular batch of horse serum is not suitable, other batches should be tried. As mentioned under "Materials and Methods," Difco's tissue culture horse serum has been found to be very good.

The test has been constructed, in some respects, by the arbitrary standardization of conditions: medium constitution, reinforcement with serum, inoculum size, incubation time, and the like. Although not ideal, and not presented as capable of indicating an absolute susceptibility to sulfonamide that would necessarily apply under conditions other than those employed in the test, the method nevertheless is capable of differentiating between strains known to be resistant and those known to be susceptible to sulfonamide on clinical and epidemiologic bases; and since that differentiation is the one for which the test is designed to be used, it can be regarded as satisfactory.

SUMMARY

A simplified method is presented for testing the in vitro resistance of group A streptococci, in which a semisynthetic powdered medium base is reconstituted with water, enriched with horse serum and mixed with sodium sulfadiazine solution in desired concentrations. The method has been shown to be able to differentiate among strains known to vary in sulfonamide sensitivity on clinical and epidemiologic grounds.

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STUDIES ON THE PROPAGATION IN VITRO OF POLIOMYELITIS VIRUSES

V. The Application of Strain HeLa Human Epithelial Cells for Isolation and Typing

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A PRECEDING paper[2] presented evidence to show that a strain of malignant human epithelial cells (strain HeLa, Gey),[3] maintained by serial cultivation in vitro since February, 1951, readily supports the multiplication of poliomyelitis virus. The propagation of each of the three types of virus was found to be accompanied by rapid (twelve to seventy-two hours) destruction of the cells. For that study, a method was developed and described for massive cultivation of the cells. This procedure permitted (a) the rapid preparation of large numbers of cultures from suspensions of cells rather than from explants, the method of cultivation used by Gey,[3] and (b) the quantitation of cellular inocula and thus the preparation of replicate cultures. The experiments demonstrated further that strain HeLa cells can easily and effectively be employed for the quantitation of virus, for the measurement of specific antibody to poliomyelitis virus by the tissue culture-neutralization technique, and for the mass production of virus.

The present investigation was carried out to learn whether cultures of strain HeLa can be used advantageously for the isolation of field strains of poliomyelitis virus and for the specific immunologic identification of such strains. Now that earlier studies[1, 2] have been substantiated by adequate development and application, it is the purpose of the present paper to describe the successive steps employed for the rapid isolation and specific immunologic identification of poliomyelitis virus from patients. This report in its presentation of evidence for the use of strain HeLa cells in culture as a practical approach to the specific diagnosis of the poliomyelitis virus infection, concerns the first 300 strains of poliomyelitis virus that were successfully isolated and identified as to immunologic type. It is apparent from this experience that the procedures as used at present in this laboratory can be applied routinely in the daily practice of medicine by resort to diagnostic and research laboratories for the isolation and identification of strains of virus, for the assessment of antibody levels in selected population groups, and for the measure-

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ment of antibody response to vaccines. For that reason, careful attention is given by the authors to details in the successive steps that have been found satisfactory for the growth of cells and for their efficient application to the isolation and identification of poliomyelitis virus from field specimens.

METHODS AND MATERIALS

Media for Growth and Maintenance of Cells.—The liquid medium to initiate cellular growth consists of 50 per cent human adult serum, 2 per cent embryonic extract and 48 per cent balanced salt solution at pH 7.4. (Hanks's solution* is used in this laboratory.) Hereafter, the designation of this medium and of similar media will be abbreviated, i.e., HAS-50, EE-2, H-48.

The medium for cellular maintenance consists either of HAS-40, H-60 or of 90 per cent maintenance solution* and 10 per cent human or chicken serum (HAS-10 or ChS-10, MS-90). The maintenance solution is essentially similar to the solution of Anfinsen and co-workers[5] as modified by Hanks[6] and Scherer.[7] Penicillin and streptomycin are employed routinely in all media for mass cultivation of cells and for viral cultivation at levels of 50 units per milliliter and 50 micrograms per milliliter, respectively.

Human serum has been found best for cellular growth. Adult serum has replaced placental serum for routine purposes. It supports cellular growth as well; it is readily available, relatively easy to collect and, finally, rarely contaminated with microorganisms. Blood, 500 ml., is withdrawn from a healthy donor into a Baxter bottle containing 0.2 per cent phenol red (2.5 ml.), but no anticoagulant. After from thirty to sixty minutes at room temperature, the stopper is removed aseptically and the clot is broken by means of a sterile pipette or glass rod. The blood is kept at 4° C. overnight preferably, or for several days, when the serum is withdrawn by volumetric pipette, 50 to 100 ml., transferred to a sterile 250 ml. centrifuge tube and centrifuged for thirty minutes at 3,000 r.p.m. The serum from each donor is distributed in aliquots for mixture with an equal quantity of serum from each of two to four other donors. Serum pools are made to avoid the use of serum deficient in antitryptic effect. The serum pool is used directly for making media, or stored at 4° C. until needed. The final 2.5 ml. of serum withdrawn from the 250 ml. centrifuge tube is used in bacteriologic tests for sterility by transfer in duplicate of 0.5 ml. (a) to two rabbits' blood agar slants, (b) to two Sabouraud's agar slants, and (c) to two tubes of thioglycollate liquid medium for incubation for seven days at 37° C. and at 20° C., respectively.

**MAINTENANCE SOLUTION (MS)*

Method for Preparation From Stock Solutions I, II, III, and IV. (The formulas and the source for purchase of ingredients are given below.)—

To prepare MS, in 100 ml. lots:

1. Mix together: 10 ml. stock I, 10 ml. stock IV, and 80 ml. distilled water (from a glass still) in a 200 ml. Neutraglas screw cap bottle (Pyrex is also satisfactory).
2. Place 7.5 ml. of stock II in a 13 x 100 mm. Pyrex test tube. Put tube in the 200 ml. bottle so that stock II does *not* mix with diluted stocks I and IV.
3. Place 2 ml. of stock III in a 13 x 100 mm. Pyrex test tube. Put tube in the 200 ml. bottle so that stock III does *not* mix with diluted stocks I and IV.
4. Autoclave the botttle, 10 lb. 10 min., with bottle cap loose.
5. After cooling, tighten cap, invert to mix all solutions in bottle, loosen cap, and place at 4°-6° C. for twenty-four to seventy-two hours to convert CO_3 to HCO_3. (Note: [a] If complete mixing of the solutions does not occur, they may be mixed by pipetting the solutions from the tubes into the solution in the bottle, just before use on cellular cultures. [b] Stock solutions II and III need not be placed within the 200 ml. bottle, but may be autoclaved separately and then mixed with the autoclaved diluted stocks I and IV. Contamination, however, may occur during mixing.)

To prepare MS in 20 ml. lots:

1. Mix together: 1 part stock I, 1 part stock IV, and 8 parts distilled water (from a glass still).
2. Place 20 ml. aliquots from step 1 in 36 ml. Pyrex (or Neutraglas) screw cap bottles.
3. Place 1.5 ml. stock II in a small test tube made from 10 mm. O.D. Pyrex tubing. Put tube in the 36 ml. bottle so that stock II does *not* mix with diluted stocks I and IV.

(Footnote continued on page 288.)

The technique employed for harvesting serum from sheep and horses is essentially similar.

Chicken serum is harvested from roosters made ready by fasting for from twenty-four to forty-eight hours. Blood, 30 ml., is withdrawn from the wing vein through a No. 20 needle into a syringe. The syringe and needle are prepared before sterilization by rinsing in 4 per cent mineral oil in ether to provide a thin film of mineral oil on the inner surfaces. The blood is transferred in 10 ml. aliquots to screw cap tubes that contain 0.2 per cent phenol red, 0.1 ml., and 20 mg. per cent heparin, 0.1 milliliter. Each tube is immediately capped, inverted to mix the contents and centrifuged for 20 minutes at 2,500 r.p.m. The plasma is withdrawn from the tubes through a long needle into a 20 to 30 ml. syringe to provide aliquots of 10 milliliters. Chicken embryonic extract, 0.1 to 0.5 ml. to clot the plasma and several milliliters of air are drawn into each syringe. Usually after several minutes at room temperature, the plasma has clotted. The needle is removed from the

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(Continued from page 287.)

4. Place 0.4 ml. stock III in a small test tube and put in the 36 ml. bottle as in step 3.
5. Autoclave the bottles, 10 lb., 10 min., with caps loose.
6. Proceed as in step 5 for the preparation of 100 ml. of MS.

MAINTENANCE SOLUTION

					Final conc. (mg./liter)
Preserve stock solutions I, II, III, and IV with $CHCl_3$, 1-2 ml./liter (slight excess).					
I.	Balanced salt solution (BSS; Hanks's) 10 x conc. For 1,000 ml. *10 x stock solution*				
	1. NaCl (Biological grade) (a)	80.0g	Dissolve the BSS salts of blocks *1* and *3* in approximately 400 c.c. glass-distilled water each and combine. Dissolve the $CaCl_2$ in 200 c.c. g.d. water and add to the above. Bring to final volume of 1,100 c.c. If necessary, filter through ash-free filter paper into a glass-stoppered Pyrex bottle.		8,000.0
	KCl (b)	4.0			400.0
	$MgSO_4 \cdot 7H_2O$ (a)	2.0			200.0
	2. $CaCl_2$ (c)	1.4			140.0
	3. $Na_2HPO_4 \cdot 2H_2O$ (a)	0.6			60.0
	KH_2PO_4 (b)	0.6			60.0
II.	$NaHCO_3$ (a) (1.4 per cent in glass-distilled water)				1,000.0
III.	Glucose (a) (10 per cent in glass-distilled water)				2,000.0
IV.	Amino acids				
	Parenamine 15 per cent with tryptophane (d)	10 ml.	in 100 ml. glass-distilled water	Combine = 500 ml. 10 × stock	300.0
	Glycine (e)	100 mg.			20.0
	Histidine (free base) – DL (e)	100 mg.			20.0
	Cystine – L (e)	75 mg.			15.0
	Glycerol (a)	2.5 Gm.	in 100 ml. g.d. H_2O		500.0
	Na pyruvate (e)	3.2 Gm.			500.0
	Na acetate·$3H_2O$ (c)	5.64 Gm.			500.0
	Succinic acid (c)	50 mg.	in 100 ml. g.d. H_2O		10.0
	l – malic (e)	25 mg.			5.0
	Adenine $SO_4 \cdot 2H_2O$ (f)	34 mg.	in 100 ml. g.d. H_2O		2.5
	Guanine $HCl \cdot 2H_2O$ (f)	8 mg.			1.0
	Xanthine (e)	5 mg.			1.0
	Uracil (f)	5 mg.			1.0
	Thymine (e)	2 mg.			0.4
	Cytosine (e)	2 mg.			0.4
	KH_2PO_4 (e)	450 mg.	in 99 ml. g.d. H_2O	100 ml.	90
	Thiamine · HCl (e)	5 mg.			1.0
	Nicotinamide (e)	2 mg.			0.4
	Ca-pantothenate (Dextrorotatory) (e)	2 mg.			0.4
	Pyridoxal · HCl (e)	2 mg.			0.4
	Pyridoxamine dihydrochloride (e)	2 mg.			0.4
	D-ribose (e)	2 mg.			0.4
	Riboflavin (e)	2 mg.			1.4
	i-inositol (e)	7 mg.			1.4
	Choline Cl (e)	7 mg.			
	Biotin (e) 1 mg. / Folic acid (e) 1 mg. / PABA (e) 1 mg.		in 20 ml. g.d. H_2O	1 ml.	0.01 / 0.01 / 0.01
	Phenol red (g) in I, II, and IV				20

(a) Merck—Reagent grade
(b) Baker's—Analyzed grade
(c) Mallinckrodt—Reagent grade
(d) Winthrop Stearns, Inc.
(e) Nutritional Biochemicals Corp.
(f) Eastman Organic Chemicals
(g) Difco Co.—T.C. grade

syringe and the clot broken by its forceful expulsion through the tip of the syringe into a test tube. Centrifugation, as previously mentioned, is repeated and the serum is transferred to tubes for storage at 4° C.

Chicken embryonic extract is prepared from nine-day embryos by the syringe method[8] modified by the use of a single stainless steel screen, 28-mesh. Embryos are removed with sterile forceps and placed in Petri dishes. From 5 to 15 embryos are transferred to the barrel of a 20 or 30 ml. syringe at the bottom of which is a circular piece of stainless steel mesh, No. 28. The plunger is put in the barrel and the embryos forced through the screen and tip of the syringe into a Rockefeller design centrifuge tube, 50 milliliters. Extraction is performed by the addition of one volume of balanced salt solution to an equal volume of tissue pulp, followed by thorough mixing and centrifugation at 2,500 r.p.m. for 20 minutes. The supernatant 1:1, or 50 per cent extract is stored in convenient aliquots at -20° C. Prior to use, the 50 per cent extract is thawed, centrifuged at 2,500 r.p.m. for 20 minutes, and only the supernatant fluid employed.

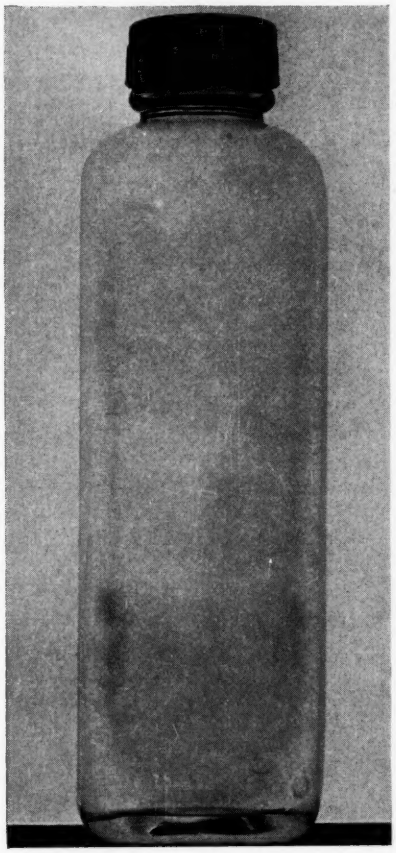

Fig. 1.

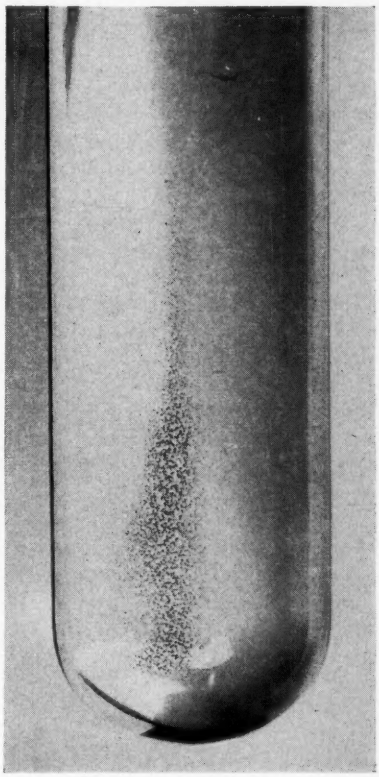

Fig. 2.

Fig. 1.—Cellular growth on the surface of the square, screw-cap bottle commonly employed for the production of cells in quantity.

Fig. 2.—Cellular growth that results on glass surface of test tube kept stationary at 36° C. for 6 days following inoculation with approximately 10,000 live HeLa cells.

Microbiologic sterility tests are carried out routinely as described for human serum.

Cellular Cultivation.—Strain HeLa cells are cultivated in vitro on a glass substrate to provide surface for cellular multiplication by using HAS-40, EE-2, H-48. The cells are cul-

tured for the production of cells in quantity in square, screw cap bottles,* 200 ml. (Fig. 1), or in larger bottles, and for test purposes in test tubes, 16 mm. (Fig. 2), stoppered by screw caps or by rubber stoppers.† The cultural vessels are incubated at 36° C. in a stationary position at a slant that permits the fluid to cover fully the cellular growth.

Bottles commonly are fed twice weekly; tube cultures seldom require feeding. The first feeding consists of the addition of 2.5 ml. of HAS-100 to the 8 ml. in each bottle, or by the addition of 0.25 ml. of HAS-80, H-20 to each tube. When subsequent feedings are indicated, feeding is accomplished by replacement of from half to two-thirds of the old medium with new medium HAS-40, H-60. After from three to fourteen days of cultivation at 36° C., more commonly seven days for tubes and ten to fourteen days for bottles, the cellular population is fully adequate for the preparation of subcultures as described above, or for the support of viral growth. The average cellular population when used for transfer or for viral cultivation is 5-8 $\times$ 10^6 for bottles and 60,000-100,000 for tubes. Whenever a cellular culture reaches maximal growth, as shown by confluent coverage of the glass surface and evidence of continued proliferation, it is removed from the 36° C. incubator. More commonly, the cells at this time are utilized immediately by transfer to bottles for increasing the supply of stock cells or to tubes for acute experiments. However, cells can be maintained satisfactorily for future use by incubation at 30° C. and feeding twice weekly. Feeding is accomplished by withdrawal of about half of the spent medium and its replacement by an equal quantity of HAS-40, H-60.

Preparation of Cells for Transfer.—HeLa cells free in suspension for transfer and other purposes are readily made available by the use of trypsin[9] to release and break up cellular sheets from glass. An inexpensive preparation of commercial trypsin, "Bactotrypsin, 1:250," as a 0.5 per cent suspension in MS-100, pH 7.4-7.6, exerts satisfactory proteolytic activity without "toxic" effects. The trypsin suspension is twice filtered by employing (a) ash-free filter paper‡, and (b) a Bush-type filter under positive pressure.§ The filtrate, upon being fast frozen and kept at -20° C., provides a standardized enzyme preparation for long periods of time. The trypsin preparation employed as routine is added to a cellular culture in a volume of 8 ml. for a bottle, or 1.0 ml. for a 16 mm. tube; whereupon the container is incubated at 36° C. for sixty to ninety minutes. The transfer to a centrifuge tube and the production of a uniform cellular suspension are effected by employing a serologic pipette to mix thoroughly until the suspension consists of single cells or groups of no more than several cells. For the preparation of pools of cells, trypsinized cells from bottles or tubes are mixed before centrifugation. Following horizontal centrifugation of the cellular suspension for ten minutes at 1,000 r.p.m., the supernatant fluid containing the trypsin is discarded and the cells are restored to volume, or greater, by adding the old medium and new nutritive medium, HAS-40, EE-2, H-58.

Cellular Enumeration.—The cells released by trypsinization and resuspended are enumerated by the transfer with a sterile capillary pipette of an aliquot of the suspension to a hemocytometer. The cells should be diluted sufficiently to avoid crowding of cells in the hemocytometer (Fig. 3). The total number of cells per cubic millimeter is calculated by the employment of the following formula:

$$\frac{\text{Total cells in four chambers (3.6 c.mm.)}}{\text{3.6 cmm.}} \times 1{,}000 = \text{Cells per cubic centimeter}$$

Upon microscopic enumeration, the cellular concentration is adjusted by the addition of fresh HAS-40, EE-2, H-58 to provide a final 100,000 cells per milliliter. (Medium from bottle cultures after three to four days of incubation may be used up to 30 per cent to conserve the supply of fresh medium.)

*Kimble Neutraglas, serum and sampling bottle.

†Pure gum rubber, West Company, Phoenixville, Pa. Rubber lined, screw caps are available from the A. H. Thomas Co., Philadelphia (size 28, Catalog No. 2849-B for bottles) and from the Kimble Glass Co. (size 15 for 16 mm. tubes).

‡Double-acid-washed; diameter, 150 mm.; S. & S. No. 589, Black ash-free from Schleicher and Schuell, or jobber.

§Filter apparatus of Bush pressure type; 40 UF fused in fritted disc with pore diameter 2; capacity 150 ml.

Preparation of Cell Cultures.—Cells in suspension in a concentration of 100,000 cells per milliliter are distributed to test tubes, 0.4 ml. per tube, or to bottles, 8 ml. per bottle. The apparatus employed for dispensing to tubes (Fig. 4) is basically similar to that devised by Evans, Earle, and associates,[10] but it has been simplified and adapted for the preparation of replicate cultures of HeLa cells en masse. It consists of an all-glass open cylinder with a delivery tube at the base (Fig. 5, *A*); a helical stirring rod (Fig. 5, *B*) operated by a ⅛ horsepower motor* with a rheostatic control keeps the cells in suspension. A piece of rubber tubing is used to protect the shaft of the stirring rod upon its attachment to the motor.

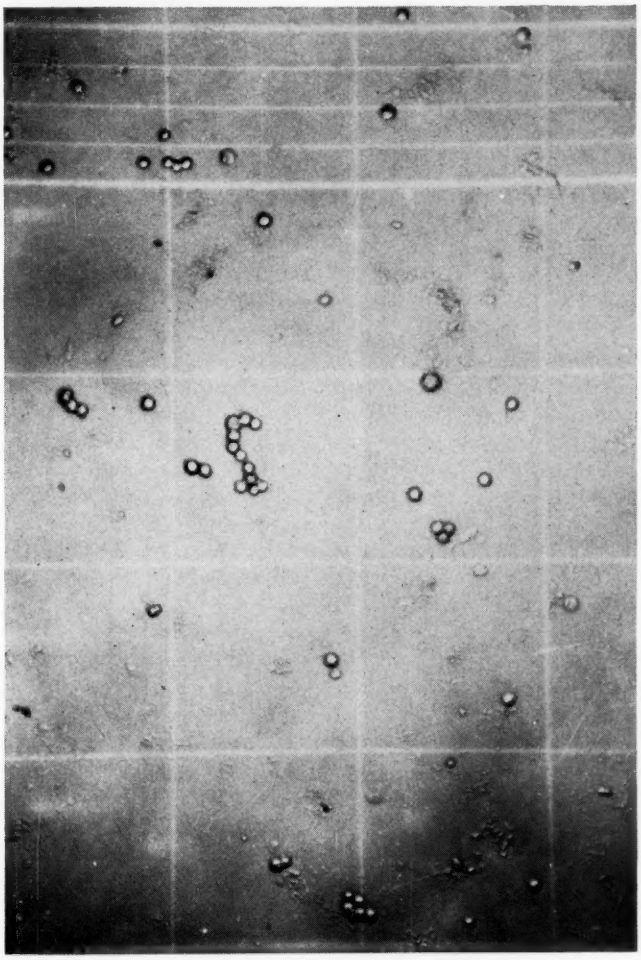

Fig. 3.—HeLa cells in hemocytometer in preparation for quantitation. Note that the rounded cells no longer present polygonal, flat, translucent appearance characteristic of HeLa cells in culture.

The cylinder is capped by a glass hood (Fig. 5, *C*) which in turn has over it a second glass hood (Fig. 5, *D*) attached by a rubber tube to the shaft of the stirring rod. A third glass hood (Fig. 5, *E*) attached by a rubber stopper or by a rubber Vacutainer stopper to the dispensing needle (Fig. 4) helps to prevent contamination. The delivery tube of the cylinder is attached to a Cornwall pipetting unit† to enable the rapid delivery of 0.4 ml.

*⅛ H.P., 115V. AC-DC, No. 7609C. Palo Laboratory Supplies, Inc., New York, or a similar gear-driven motor.

†Cornwall pipetting unit, Becton, Dickinson & Co., Rutherford, N. J.

aliquots to test tubes, 16 × 150 mm. The tubes are stoppered and placed horizontally immediately to permit the cells to settle out on the glass surface. Cultures are kept stationary at 36° C. during the growth period and for maintenance at 30° C.

Shipment of Cells.—The ability of strain HeLa cells in culture to survive shipment by ordinary channels for delivery by air mail and railroad is established.[2] More recently, bottles, tubes, and flasks have been sent to all parts of this country and abroad with success.

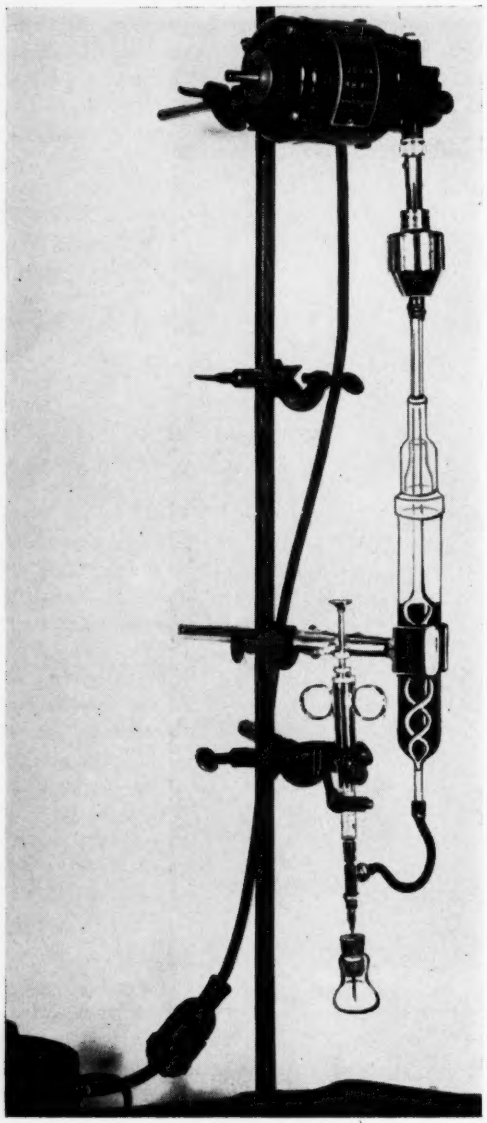

Fig. 4.

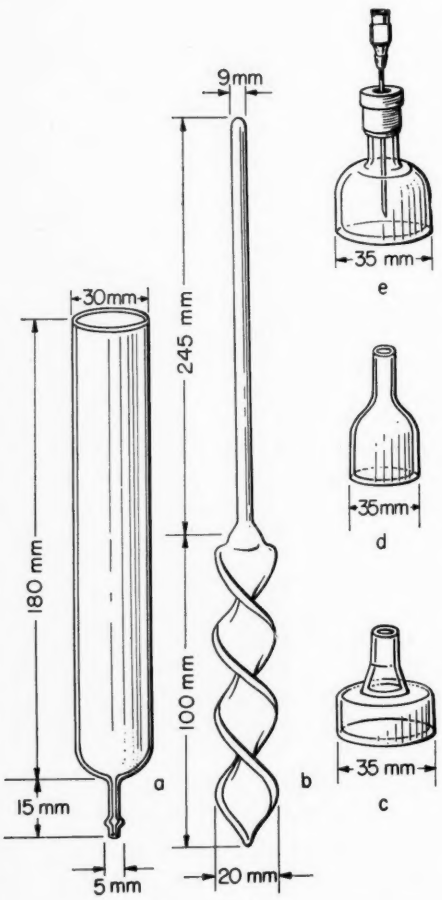

Fig. 5.

Fig. 4.—Apparatus assembled for dispensing replicate samples of a cellular suspension.

Fig. 5.—Reservoir and stirrer assembly: (*A.*) the reservoir for the cellular suspension during delivery consists of an open, glass cylinder with a delivery tube; (*B.*) helical glass stirring rod for keeping cells in even suspension; (*C.*) glass cover for cylindrical reservoir to prevent contamination; (*D.*) second cover which is attached to stirring rod; (*E.*) glass hood attached to the Cornwall pipetting unit to facilitate the aseptic dispensing of a cellular suspension to tubes.

Bottles for shipment over a three- to four-day period should contain at least 3-4 × 10^6 healthy cells, tubes from 60,000 to 100,000 cells. The overlying medium (HAS-40, H-60) is removed before shipment and transferred to a glass container which accompanies the cells. It is desirable to minimize the time in transit and to avoid exposure of the cells to temperatures above 38° C. We have found shipment by air mail, special delivery, the most effective means for rapid delivery, although many shipments were made by parcel post, special handling. Upon arrival at the destination, the cells must be revived before use. Revival of the cells usually occurs upon replacement aseptically of the medium that was sent with the cells and reincubation at 36° C. for twenty-four to forty-eight hours. If cells in tubes are shipped without liquid, approximately 0.25 ml. is transferred with sterile equipment, e.g., a 5 ml. syringe and 3 inch No. 22 needle, to each cultural tube. The cells are kept covered with a layer of fluid during incubation by slanting the glass container. No rack has yet been standardized. If restoration to normal cellular morphology is not complete, cells are fed by adding HAS-40, or 50, H-60 or 50. Cultures should be attended immediately upon arrival. When circumstances on the day of arrival prevent care of the cultures, the shipping container is kept at from 22° to 25° C. (room temperature) until the following morning. When microscopic observation at twenty-four-hour intervals has established normal cellular morphology as evidenced by cells that are polygonal, flat, and translucent, with clearly visible nuclei, nucleoli, cytoplasmic processes, and surface folds, the cellular cultures may be employed as indicated.

Preparation of the Test Inoculum.—The procedure employed as routine is applicable to any material containing a mixed microbial flora. It depends on differential centrifugation and antibiotics to rid extraneous bacterial and fungi from material suspected of containing virus. As applied to a stool specimen, 4 Gm. of feces are transferred to a screw cap 125 ml. bottle that already contains 46 ml. of sterile glass-distilled water and 2 mg. per cent phenol red. The indicator by reflecting extraordinary changes in pH quickly reveals many of the specimens that prove "toxic." Morover, as routine, a second fecal specimen for virus isolation is obtained from each patient. The bottle is sealed, placed on a mechanical shaker and subjected to 450 r.p.m. for 30 minutes at 4° C. The content is transferred to a 40 ml. Spinco plastic tube for centrifugation in a Spinco preparative angle centrifuge for one hour at 15,000 r.p.m. (19,600 × G). The sediment is discarded; penicillin, 500 units, and streptomycin or dihydrostreptomycin, 250 μg are added to the clear supernatant fluid and it is ready to test for virus (Test Sample A).

The virus contained in a test sample can be concentrated and rid from the extraneous debris contained in the fluid by the use of greater centrifugal force. For example, the procedure employed for the first 100 strains of poliomyelitis virus that were isolated in this laboratory included the use of centrifugal force as described above to yield Test Sample A and, in addition, to subjecting the supernatant fluid to further treatment for the production of Sample B. Thus, following centrifugation for forty-five minutes at 15,000 r.p.m., the supernatant fluid from each 40 ml. Spinco plastic tube was decanted to a 12 ml. Spinco tube for angle centrifugation in a Spinco No. 40 head for sixty minutes at 39,000 r.p.m. (106,000 × G). The supernatant fluid that resulted therefrom was discarded; the sediment was taken up in 1.5 ml. of sterile water; penicillin and streptomycin or dihydrostreptomycin were added to yield for each a final concentration of 250 units per milliliter. The resultant virus suspension thereupon was ready for transfer to labeled glass ampules for test and/or for storage.

Method for Virus Isolation and Cultivation.—The following procedure is recommended as routine for each test material:

Two tubes with moderately heavy cellular growth (60,000-100,000 cells), commonly from five to ten days old, are selected for each sample and labeled accordingly. The nutritive medium is removed from each tube by using a pipette or long needle attached to suction and replaced by 0.9 ml. MS-100 to wash the cells and wall of the tube. Scraping of cells from the glass should be carefully avoided. An hour later a second replacement is made with 0.9 ml. ChS-10, MS-90 to ensure adequate dilution of any antipoliomyelitis anti-

body in the original nutritive medium. Washing the cells twice to remove antibody by dilution obviously is unnecessary when the serum employed for the growth of cells is known to be free from antiviral substances. ChS-10 or HAS-10, MS-90, 0.5 ml. is adequate for maintaining normal morphology of approximately 60,000 to 100,000 cells at 37° C. for from four to seven days; 1.0 ml. of medium usually suffices for about ten days. The test inoculum, 0.1 ml., is added and the tubes are placed at 36° C. Microscopic examination daily reveals the presence of virus by the rapidly progressive cellular destruction that results from infection. When cellular destruction occurs, commonly in from twelve to seventy-two hours, the viral cultures are available for typing. When the time requisite, however, for the cultivation of virus or other test material exceeds five to seven days, the supernatant fluid is replaced by maintenance solution containing 10 per cent chicken serum.

Cytopathogenicity.—The rapidity and the extent of cellular degeneration provide an index for the infection of HeLa cells by poliomyelitis virus. This cytopathogenic effect of poliomyelitis virus[11] is entirely comparable to the sequence of changes that has been described for the infection by poliomyelitis virus of other cells. The cultures are observed for cellular changes in from eighteen to twenty-four hours after infection, and thereafter daily for not more than seven days. If virus be present, total degeneration is observed in from eighteen hours to six days, commonly in from twenty-four to seventy-two hours. The extent of degeneration is readily observed microscopically by employing a 10× wide field ocular, and either a 5 N.A. 0.14 objective, or, less commonly, a 10 N.A. 0.25 objective for direct observation of the cellular growth on the glass wall of the container. The changes observed are recorded according to a standard scale:

0 = No change.

1+ = Early degeneration of epithelial cells. The cells may be rounded and transparent; the cells situated peripherally may be swollen, undergoing disintegration and partially released from the glass. Similar changes may occur in cultures from three to seven days old as the result of nutritional or other disturbance.

2+ = The cells show evidence of degeneration as shown by distortion, swelling, or shrinkage, total loss of intercellular bridges eversion, and release from the glass; the nuclei are karyorrhectic or pyknotic.

3+ = Cells for the most part are totally degenerate with some persisting as round granular distorted cells with pyknotic nuclei and loss of staining affinities.

4+ = Dead cells that either slough from the glass wall or remain as shrunken granular cell shadows.

Preparation of Type-Specific Antiserum.—Specific antiserum to each of the three immunologic types of poliomyelitis virus is obtained from 3 to 6 cynomolgus monkeys which have survived intracerebral injection and/or hyperimmunization. The virus samples employed as antigen are laboratory strains, representative of the three prototypes. The descriptive data for these strains have been presented previously.[2, 12] Hyperimmunization is effected by the use of adjuvants, as recommended by Salk and co-workers.[13] The antigent as tissue culture fluid with a TCD_{50} in a range from 10^5 to 10^6 is prepared in emulsion by adding an equal amount of adjuvant mixture (9 parts Bayol F* and 1 part Arlacel A.†) The resultant emulsion is injected intramuscularly fortnightly in three doses, 0.25 ml., 0.5 ml., 1.0 ml. At intervals of from two to four weeks, immediately following each bleeding 1.0 ml. booster doses are given. The resultant antiserum protects HeLa cells in culture from infection with from 10^4 to 10^6 TCD_{50}. Dr. H. A. Wenner kindly made available for control purposes samples of the type-specific poliomyelitis antisera which had been utilized by the Committee on Typing of the National Foundation for Infantile Paralysis.[14]

Method for Immunologic Identification of Cytopathogenic Strains of Virus.—Each unknown field strain of cytopathogenic virus is allocated as to immunologic type of poliomyelitis

*Obtained through the courtesy of Mr. C. L. Wentz, Penola Oil Co., Detroit, Mich.
†By courtesy of Mr. C. D. Pratt, Atlas Powder Co., Wilmington, Del.

virus by employing hyperimmune monkey sera representative of each of the three known types of poliomyelitis virus. For each test, 8 tubes containing 60,000 to 100,000 HeLa cells (five to ten days old) are employed. The procedure stepwise follows: (a) to provide for dilution of any antiviral substance in the nutritive fluid, the fluid is removed from each tube and replaced within a few minutes by 0.9 ml. H-100 followed by 0.9 ml. ChS-10, MS-90 or MS-100; (b) the 8 tubes are labeled with patient's name and number for identification; (c) the content of each tube within thirty to sixty minutes after the second washing is replaced by maintenance solution containing 10 per cent monkey serum. For control purposes, normal monkey serum is placed in tubes 1 and 2, antipoliomyelitis Type 1 serum in tubes 3 and 4, Type 2 antiserum in tubes 5 and 6, and Type 3 antiserum in tubes 7 and 8. The tubes are observed microscopically daily. Commonly in the presence of poliomyelitis virus, the cells in 6 of the 8 tubes are destroyed in from one to four days and rarely on the fifth or sixth day. Accordingly, in the absence of cytopathogenic changes, the final reading is not made until the seventh day. The unaltered cells in two of the tubes reflect the protective or neutralizing effect of one of the type-specific antisera, and thereby indicate for the test strain of virus its immunologic type.

RESULTS

The procedures described for the culture of strain HeLa human cancer cell on glass surfaces kept stationary were applied successfully to the isolation of poliomyelitis virus and to the immunologic differentiation of each strain as Type 1, 2, or 3. The specimens employed for the isolation of virus were feces, nasopharyngeal washings, and/or brain and cord. The data and results are given in Table I.

TABLE I. SUMMARY OF THE RESULTS OF EMPLOYING CULTURES OF HUMAN EPITHELIAL CELLS (HeLa) FOR THE ISOLATION AND IMMUNOLOGIC CLASSIFICATION OF POLIOMYELITIS VIRUSES: DISTRIBUTION OF IMMUNOLOGIC TYPES AMONG FIRST 200 ISOLATES

SPECIMEN FROM	TOTAL BY YEAR	IMMUNOLOGIC TYPE		
		T1	T2	T3
1946	7	7	0	0
1948	9	9	0	0
1949	10	4	6	0
1950	54	21	1	32
1951	14	11	2	1
1952	85	77	0	8
1953	121	118	1	2
Total	300	247	10	43
Percental Number		82	3	14

The results of allocation by immunologic type of the strains of poliomyelitis virus which were isolated from 300 patients over a period of eight years are given in Table I. These patients had been admitted to hospitals in Minneapolis or Iowa City (3 patients) with a clinical diagnosis of poliomyelitis, paralytic, or nonparalytic. The frequency of occurrence of the three basic types of poliomyelitis virus varies with the year of isolation.

The percental distribution by types shows that Type 1 predominates over all (82 per cent) and annually except for 1950. These composite findings suggest for this north central region of the United States that Type 1 (Brunhilde) virus was mainly responsible for clinical poliomyelitis over a period of seven years. Type 3 (Leon) infection prevailed, however, in a ratio of 3 to 2 for

1950 and Type 2 was not uncommon in 1949. Finally, it should be noted that the numbers of strains isolated in 1946,[7] 1947,[6] 1948,[9] and 1949[10] are insufficient to provide significant information concerning the annual distribution of each virus type.

Fecal suspensions may contain material of varying toxicity for cells in culture. We have found that the degree of toxicity varies by daily sample and by patient. Our experience was similar to that recorded by Youngner and co-workers,[15] when two explants of HeLa cells were employed for isolation of virus from feces. Not uncommonly the explants by sloughing from the glass wall were made useless. This problem was minimized by the employment of confluent cellular growth on glass as described in this paper. Nevertheless, for the first hundred strains of poliomyelitis virus that were isolated and typed, the procedure already described, and now employed as routine, was extended to provide for each test material a second sample (Test Sample B). This second sample was prepared by subjecting the supernatant fluid that resulted from angle centrifugation for forty-five minutes at 15,000 r.p.m. (19,600 × G) to angle centrifugation for 60 minutes at 39,000 r.p.m. (106,000 × G). The resultant sediment was resuspended in 1.5 ml. of sterile glass distilled water. It was expected that this procedure would (a) materially lessen toxic effects and (b) result in recovery of virus from specimens that proved negative upon a single centrifugation. When it was found upon review of the results obtained from the study of 100 successive isolations of virus that the procedures had failed to accomplish any of these three objectives, resort to a second centrifugation was discontinued.

In our experience the undesirable "toxic" specimens have more commonly resulted from the use of enemas, particularly soapsuds, or from any of a variety of antiseptics that may be employed in cleansing bedpans. The problem has been helped, in part, by a directive requesting care in procurement of specimens, the immediate transfer to minimize bacterial growth to an ice chest at 4° C. for overnight storage or to –20° C. for longer storage. A most effective and simple measure, however, is the use routinely of glass-distilled water containing 2 mg. per cent phenol red as the diluent for suspending feces. A fecal suspension made up in this diluent reflects immediately by an abrupt change in pH, particularly excessive acidity of unknown cause, "toxicity," and thereby the need for a second test specimen. This simple expedient and the immediate availability of a second fecal specimen by requesting routinely two fecal samples from each patient have resolved the problem.

It is not the purpose of this paper to relate the available data by year of isolation to the clinical type of illness, the geographic origin, or the source. These clinical and epidemiologic findings will be reported in a second communication when the findings are available from the large number of virus isolations and typings that are resulting for the 1953 season.

DISCUSSION

In the study reported here, a strain of human epithelial cancer cell, strain HeLa, kept in continuous serial passage for more than two years, was found to

excel in its application to the isolation of strains of virus directly from patients and to the immunologic differentiation of these strains into the three basic serologic types of poliomyelitis virus. Three hundred field strains of virus were successfully allocated as to type.

The advantages of the employment of this cell strain for the laboratory diagnosis of poliomyelitis are many. It grows abundantly to yield in from four to ten days sufficient cells for the propagation of virus from field specimens or for other purposes. The cells respond to infection by poliomyelitis viruses with rapidly progressive degeneration and death. Conversely, the destructive effects of each viral type are specifically and completely inhibited by the homotypic antibody to result upon the neutralization of virus by antibody in the persistence of normal-appearing cells in cultures. Thus the protocol described for typing was used both for isolation and for typing as a single procedure to result in a rapid, specific diagnosis.

The results of previous studies concerned with the immunologic classification of poliomyelitis viruses will be cited for comparative purposes.

The series of papers which were sponsored by the Committee on Typing of the National Foundation for Infantile Paralysis[14] provided, for the first time, information concerning the immunologic classification of a significant number of strains of poliomyelitis virus. The cooperative efforts of this group of workers made available the type distribution of 196 strains, collected over a period of forty-three years, 1909 to 1951. More recently, at least four laboratories have applied tissue culture techniques to the immunologic classification of poliomyelitis virus. Thus, 86 poliomyelitis viruses were isolated and established by type at the Children's Medical Center, Boston.[16-18] Riordan and associates[19] studied 21 isolations, Youngner and co-workers[15] reported 37 isolations, and Syverton and Scherer[1] recorded, preliminary to the present study, the recovery and typing of 40 strains. Accordingly, by including the 300 strains reported herewith, 591 strains (Table II) are now available for comparison of the distribution of the three prototype viruses by geographic origin, annual occurrence, year of isolation, source and nature, frequency distribution by type of clinical disease and other information of importance to an understanding of the recurrence annually of epidemic poliomyelitis. This number undoubtedly will be vastly increased by studies under way to provide sufficiently large samples from assorted, selected geographic areas and environments. It is apparent from consideration of the meager accumulative data to date that many comprehensive studies are indicated to learn for a wide variety of populations the seasonal recovery of virus and antibody response. These studies are made readily possible by the recent development of tissue culture techniques and by their application for rapid isolation and typing of poliomyelitis viruses and for assay of antibody.

The first 200 strains isolated in our laboratory provides a collection representative of a period of eight years, 1946 to 1953. This collection is reasonably comparable in number and occurrence to the 196 strains reported by the Com-

TABLE II. SUMMARY OF TYPE DISTRIBUTION FOR 591 STRAINS OF POLIOMYELITIS VIRUS COLLECTED OVER A FORTY-FOUR-YEAR PERIOD, 1909-1953

YEAR	TOTAL	DISTRIBUTION BY IMMUNOLOGIC TYPE					
		T1 (BRUNHILDE)		T2 (LANSING)		T3 (LEON)	
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
1953	121	118	99.0	1	1.0	2	
1952	138	105	83.4	8	5.8	15	10.8
1951	27	22	81.4	2	7.4	3	11.2
1950	108	48	44.4	3	2.7	57	52.9
1949	47	35	74.5	11	23.4	1	2.1
1948	46	42	91.3	3	6.5	1	2.2
1947	17	14	81.7	2	12.4	1	5.9
1946	25	22	88.0	2	8.0	1	4.0
1945	11	10	90.9	0	-	1	9.1
1940-1944	40	31	77.5	3	7.5	6	15.0
1934-1939	10	5	50.0	4	40.0	1	10.0
1909-1914	1	0	-	1	100.0	0	-
Total	591	452	71.84	40	17.89	89	10.26

mittee on Typing.[14] The latter group had been collected from 1909 through 1951; however, 145 were representative of the years 1945 to 1951. These strains were diverse in geographic origin, natural source, clinical category, and year of occurrence. For example, 164 of the strains were from all parts of the United States and the remaining 32 from different countries representative of all parts of the world. This cosmopolitan geographic origin is in striking contrast to the present series which was obtained from patients hospitalized in Minneapolis, St. Paul, or Iowa City (3 patients). These patients, for the most part, were from Minnesota; a few were from Wisconsin, the Dakotas, Iowa, or elsewhere. These two studies make available for review the type distribution of about 400 strains of poliomyelitis virus. A comparative tabulation of the percental incidence of types by year for these two studies is presented in Table III.

TABLE III. COMPARATIVE ALLOCATION BY TYPES OF THE 200 STRAINS OF POLIOMYELITIS VIRUS ISOLATED BY THE AUTHORS AND THE 196 STRAINS CLASSIFIED IMMUNOLOGICALLY BY THE COMMITTEE ON IMMUNIZATION OF THE NATIONAL FOUNDATION FOR INFANTILE PARALYSIS

TYPE	STRAINS TYPED	
	NUMBER	PER CENT
1	149 (161)	74.5 (82.1)
2	10 (20)	5.0 (10.2)
3	41 (15)	20.5 (7.7)
Total	200 (196)	

It can be seen from the data in Table IV relating to 396 strains that Type 1 predominated in occurrence. Nevertheless, Types 2 and 3 were not uncommon. For example, in the authors' series of the first 200 strains typed, 20 per cent were Type 3, and 5 per cent were Type 2. The accumulative incidence of Type 2 and of Type 3 approximates that for the Committee on Typing. It commonly is assumed that infection by Type 3 virus is comparatively rare. This assumption resulted from the rarity of isolation in years prior to 1950. In contrast to this assumption the authors' series included 41 strains in Type 3.

It can be seen that Type 3 predominated by 3 to 2 for 1950 (32 of 54 strains), whereas only 9 of 109 strains collected during the years 1949, 1951, 1952, and 1953 were in Type 3. Despite the evidence from the collective findings of the Committee on Typing that Type 3 infection occurs rarely, the high incidence for 1950 of Type 3 infection was not limited to the north central area. Indeed, the latter study substantiates the findings of Robbins, Enders and co-workers,[16-18] who reported the isolation in Boston of Type 3 virus from 10 of the 11 poliomyelitis patients studied in 1950. Similarly, Youngner and associates,[15] in their report on the classification by tissue culture studies for 37 strains which had been isolated in 1949, 1950, and 1951, showed that 28 strains were of Type 1 virus, and 9 strains were of Type 3 virus. Since the authors did not indicate the isolates by year of occurrence, the findings were not included in Table III. Riordan, Ledinko, and Melnick,[19] on the other hand, found in 1950 only three Type 3 viruses of the 16 strains isolated in Easton, Pennsylvania.

TABLE IV. DISTRIBUTION BY YEARS OF ISOLATION OF THE STRAINS OF EACH TYPE AMONG FIRST 200 ISOLATES AT UNIVERSITY OF MINNESOTA

SPECIMEN FROM	TOTAL BY YEAR	IMMUNOLOGIC TYPE		
		T1	T2	T3
1909-14	(1)*		(1)	
1034-39	(10)	(5)	(4)	(1)
1940-44	(40)	(31)	(3)	(6)
1945	(11)	(10)		(1)
1946	7 (17)	7 (14)	0 (2)	0 (1)
1947	(29)	(28)	(1)	(0)
1948	9 (36)	9 (32)	0 (3)	0 (1)
1949	10 (28)	4 (22)	6 (5)	0 (1)
1950	54 (16)	21 (12)	1 (1)	32 (3)
1951	14 (8)	11 (7)	2 (0)	1 (1)
1952	85	77	0	8
1953	21	20	1	0
Total	200 (196)	149 (161)	10 (20)	41 (15)
Per cent		75 (82)	5 (10)	20 (8)

*No. in ( ) = Distribution 196 strains typed by Committee on Immunization, N.F.I.P.

It is at least evident from the information to date that generalizations should not result from single studies even though the study is as comprehensive as that designed by the Committee on Typing.[14]

The usefulness of cells in tissue culture for the isolation and typing of poliomyelitis virus has been demonstrated for human cells derived by primary explants from adult and fetal tissues[16-18] and for monkey cells derived by explants from testes and kidneys.[15, 19] A comparative evaluation of the results on record for other cellular systems seems to emphasize that the procedures described herein for the practical employment of strain HeLa cells for the isolation and typing of field strains excel in convenience, economy, and rapidity. Moreover, this stable strain of human cell provides a uniform host cell which reflects in from twelve to ninety-six hours its susceptibility by progressive degenerative alterations that result in total destruction. The growth of cells in the absence of plasma on a glass surface kept stationary makes easy the observation of the rapidly destructive changes to total destruction that

results from infection by poliomyelitis virus. The multiplication of HeLa cells to provide a five- to tenfold increase in from five to ten days provides a heavy cellular growth. Moreover, the capacity of an adequate growth of cells to withstand almost regularly the immediate "toxic" effects of feces in suspension facilitates the successful isolation and typing of a poliomyelitis virus as a single step. The comparative rapidity of the test is brought out by citing the experience of Youngner and his associates.[15] These investigators employed roller tube cultures of monkey testicular cells for the isolation of 37 strains from fecal specimens collected from 51 patients in the years 1949, 1950, and 1951. The time required by this procedure for isolation and typing was from six to eighteen days and commonly required twelve days. Monkey kidney cells by more rapid response to infection are reasonably comparable to the HeLa strain cells. It will be of interest in the future to compare the results of studies that utilize the two cell types.

SUMMARY

A stable strain of human epithelial cancer cell (strain HeLa, Gey) has been under study in this laobratory for more than a year. During that time, it has been established[2]: (a) that this strain of human cells supports the growth of poliomyelitis virus and a variety of other viruses[20]; (b) that these cells growing in sheets on glass surfaces in the absence of plasma are readily released by trypsin to provide single cells free in suspension; (c) that cells in suspension can be quickly enumerated by counts in a hemocytometer and readily dispensed by a simple apparatus in aliquots to provide large numbers of replicate cultures or to provide single cells for intimate host cell-virus studies; (d) that the cells settle and stick to glass walls kept stationary at 36° C. in a nearly horizontal position to reproduce by five- to tenfold in five to ten days; (e) that the stability of these cells while in continuous serial passage makes it apparent that the strain can be maintained in perpetuity by continuous transfer in series; (f) that the capacity to grow in fluid media on glass has simplified cultivation; (g) under the extraordinary environmental conditions that result from shipment by train and plane, these cells possess hardiness and a capacity for renewed proliferation upon adding nutrient media.

A recognition of the importance of learning whether the HeLa strain of cells can be employed as a practical means for the rapid laboratory diagnosis of acute poliomyelitis and for the specific allocation of field strains by immunologic type led to the isolation of 300 strains of poliomyelitis virus from 300 patients admitted with a clinical diagnosis of poliomyelitis to hospitals in Minneapolis and Iowa City. The specimens utilized for study consisted of feces, throat washings, and brain or cord tissue. Virus commonly was isolated in from twenty-four to ninety-six hours. Moreover, by employing a protocol that combined isolation and typing directly from the fecal specimen, a specific diagnosis as to type was effected in a single step.

The specimens that were employed for study were representative of the outbreaks for 1946, 1948, 1949, 1950, 1951, 1952, and 1953. The percental dis-

tribution of the strains by immunologic type were: Type 1, 74.5 per cent; Type 2, 5 per cent; Type 3, 20.5 per cent.

The results of the present study and the findings of the previous studies[1, 2] make it apparent that cellular cultures can be prepared in a central laboratory and distributed as needed to diagnostic laboratories throughout the country (a) for the identification of strains of poliomyelits virus, (b) for the assessment of antibody levels for selected population groups, and (c) for the measurement of antibody response to vaccines.

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THE HEMOLYTIC EFFECT OF PRIMAQUINE

I. The Localization of the Drug-Induced Hemolytic Defect in Primaquine-Sensitive Individuals

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OF THE current curative antimalarial agents, primaquine has the highest chemotherapeutic ratio. It shares, however, with several other members of the 8-aminoquinoline series, the property of inducing an acute hemolytic anemia in a small group of susceptible recipients.[1, 2] A mild anemia is not infrequently observed in Caucasians[3] during the administration of primaquine, but acute crises are found almost exclusively in the more heavily pigmented races. Hockwald and co-workers,[4] for example, observed 5 cases exhibiting acute hemolysis in a group of 110 male American Negroes receiving 30 mg. of primaquine base daily. The same investigators demonstrated that an individual who is sensitive to primaquine is also sensitive to certain other 8-aminoquinolines. Occasional reports of acute hemolysis occurring in Caucasians who were receiving an 8-aminoquinoline have appeared.[2, 5, 6] Usually, in such circumstances, quinine or quinacrine was concurrently administered and, in many instances, the drug combination was employed in the treatment of infection due to P. *falciparum*. Some of these cases, therefore, may have been examples of blackwater fever. At present, the role of drug toxicity per se in the etiology of blackwater fever is not clear. It is probable that the hemolytic crisis induced by certain 8-aminoquinolines, when uncomplicated by the administration of other drugs or by malaria, is a phenomenon almost exclusively limited to a few susceptible members of heavily pigmented races. Primaquine-sensitive men are otherwise healthy individuals and, except for an unusual response to certain 8-aminoquinolines, have no known hematologic abnormality.

This is the first of a series of studies on the mechanism of the primaquine-induced hemolytic reaction. The subject of this paper is the localization of the drug-induced abnormality to either (a) a unique susceptibility of the erythrocyte or (b) extracorpuscular factors developed in response to the administration of primaquine.

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This work was further aided by the participation of Army Medical Officers assigned to the project by the Surgeon General of the United States Army.

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METHODS

This study consisted of a series of cross-transfusions of radiochromium* (Cr^{51}) labeled erythrocytes between primaquine-sensitive and normal subjects. The fate of the tagged cells was determined before and during administration of the drug to the recipients.

Subjects.—All men used in this study were volunteers from the Illinois State Penitentiary at Stateville with the exception of one University of Chicago student. All "primaquine-sensitive" men were healthy American Negroes, none of whom had ever had malaria. The term "primaquine-sensitive" refers to the development of the hemolytic reaction and does not imply a sensitization in the immunological sense. The primaquine nonsensitive men will be designated as "normal subjects" in this paper. The normal men, including both Caucasians and Negroes, were previously studied extensively to establish their hematologic stability during the administration of the drug. The student was chosen for study because he had had a splenectomy for traumatic rupture of the viscus two years previously but, in other respects, was normal.

Transfusion Compatibility.—Recipients for transfusion were accepted only if they were compatible with the donor in respect to the following factors: A, B, Rh_0 (D), rh′ (C), rh″ (E), and Hr_0 (c). Final cross-matching was done by both the conventional saline cell suspension and the Coombs' techniques.

Labeling and Counting Technique.—The red cells were labeled with radioactive sodium chromate ($Na_2\ Cr^{51}\ O_4$) according to the method of Gray and Sterling.[7] Details of the exact procedure have been given by Necheles, Weinstein, and LeRoy.[8] Blood was collected from the donor in amounts adequate to provide the desired number of 70 ml. aliquots for transfusion into each recipient. Radioactive sodium chromate solution was added to the heparinized blood in amounts to provide from 200 to 300 microcuries per aliquot, and the mixture was incubated at room temperature for one hour. The cells were then washed twice with physiologic saline and the blood was reconstituted with saline to approximately the original volume. A 70 ml. aliquot of the labeled cell suspension was injected into each recipient. Blood samples drawn at intervals of several days were heparinized and 3 ml. specimens, in duplicate, were counted in a 7 ml. standard Kahn tube in a well-type scintillation counter. Each duplicate was counted for two one-minute periods and corrected for background. Correction for decay of the isotope was made by dividing the average count from each blood sample by a simultaneous count from a Cr^{51} standard prepared at the beginning of the experiment. Each corrected count was a measure of the percentage of injected cells remaining in the recipient's circulation at the time of withdrawal of the sample.

Necheles and co-workers[8] have presented evidence that the determination of the "apparent survival curve" by the Cr^{51} method is equivalent to the measurement of the actual survival of red cells as a function of time. To obtain a curve for the true survival time, the data must be corrected for the known rate of elution of Cr^{51} from the tagged cells. In this paper, the term "survival curve" will refer to the Cr^{51} "apparent survival curve."

Procedure.—In the first set of studies (Group I), blood was drawn from three primaquine-sensitive Negroes and the erythrocytes were labeled as described previously. Aliquots of the labeled blood from each donor were injected into separate groups of normal men, both Negro and Caucasian, and reinjected into the donor. During a control period of from four to ten days, daily samples of blood were drawn and the percentage of labeled cells remaining in each was determined. Approximately one-half of the group was then given primaquine orally, 30 mg. base daily, for from seven to fourteen days. Samples of blood were drawn at intervals of from one to four days for the duration of the experiment. A survival curve of the red cells being tested was prepared from the determinations of the fraction of labeled cells persisting in each sample.

In the second set of experiments (Group II), the red cells of normal men were labeled and injected into other normal men and into primaquine-sensitive Negroes. Similarly, after a control period, 30 mg. of primaquine base was administered daily to one-half of the group. Frequent blood samples were again studied as described previously.

*The radiochromium used in this study was obtained from The Abbott Laboratories under an authorization from the Isotopes Division, United States Atomic Energy Commission, Oak Ridge, Tenn.

RESULTS

Group I. Cells From Primaquine-Sensitive Men in Normal Recipients.— The fate of labeled red blood cells from three primaquine-sensitive Negroes when transfused into groups of normal recipients is illustrated in Figs. 1, 2, and 3. Each figure represents an experiment with a different primaquine-sensitive donor. Prior to the administration of primaquine, all survival curves of the transfused cells had similar slopes. When 30 mg. primaquine base was given daily, the slope of the survival curves increased sharply in both Negro and Caucasian recipients, indicating hemolysis of the injected cells. In contrast, the slope of the survival curves in control recipients, who were not given primaquine, remained unchanged. No significant difference was noted between the survival curves of the transfused cells in the splenectomized and the other recipient (Fig. 3).

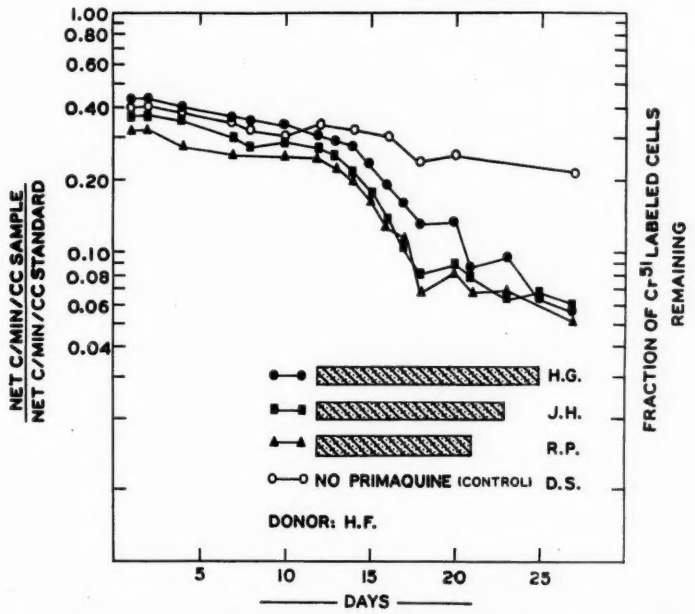

Fig. 1.—The fate of red blood cells from a primaquine-sensitive volunteer when transfused into normal (nonsensitive) men who were then given primaquine, and into suitable controls. Solid symbols: primaquine, 30 mg. base daily for the period indicated by the hatched block. Hollow symbols: no drug. H. G., D. S., and R. P.: Caucasian recipients. J. H.: normal Negro recipient. Donor H. F.: primaquine-sensitive Negro.

The percentage of labeled cells which hemolyzed could be estimated from the data of Figs. 1, 2, and 3. For example, after seven days of drug about 40 to 60 per cent of the cells were destroyed. This figure approximated the degree of anemia observed when the drug was administered to primaquine-sensitive men for seven days.

There was no change in hemoglobin concentration, hematocrit, serum bilirubin, serum iron, or fecal urobilinogen in any of the men during the course of the experiment. The Coombs' test was negative in all subjects at the beginning and at the end of the experiment.

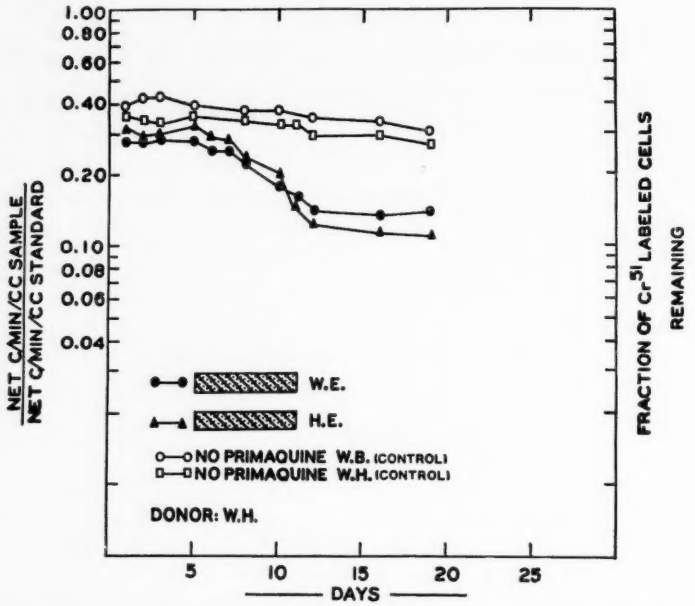

Fig. 2.—The fate of red blood cells from a primaquine-sensitive volunteer when transfused into normal (nonsensitive) men who were then given primaquine, and into suitable controls. Solid symbols: primaquine, 30 mg. base daily for the period indicated by the hatched block. Hollow symbols: no drug. H. E.: Caucasian recipient. W. E. and W. B.: normal Negro recipients. W. H.: primaquine-sensitive Negro recipient. Donor W. H.: primaquine-sensitive Negro.

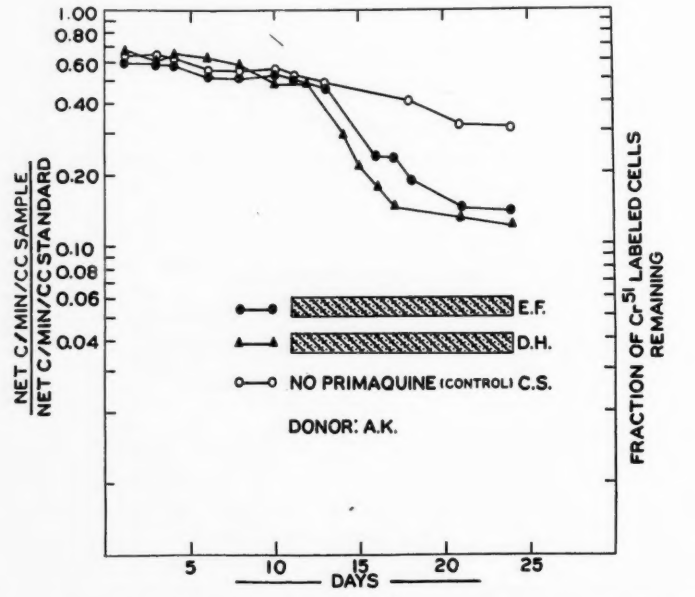

Fig. 3.—The fate of red blood cells from a primaquine-sensitive volunteer when transfused into normal (nonsensitive) men who were then given primaquine, and into suitable controls. Solid symbols: primaquine, 30 mg. base daily for the period indicated by the hatched block. Hollow symbols: no drug. E. F. and C. S.: Caucasian recipients. D. H.: splenectomized Caucasian recipient. Donor A. K.: primaquine-sensitive Negro.

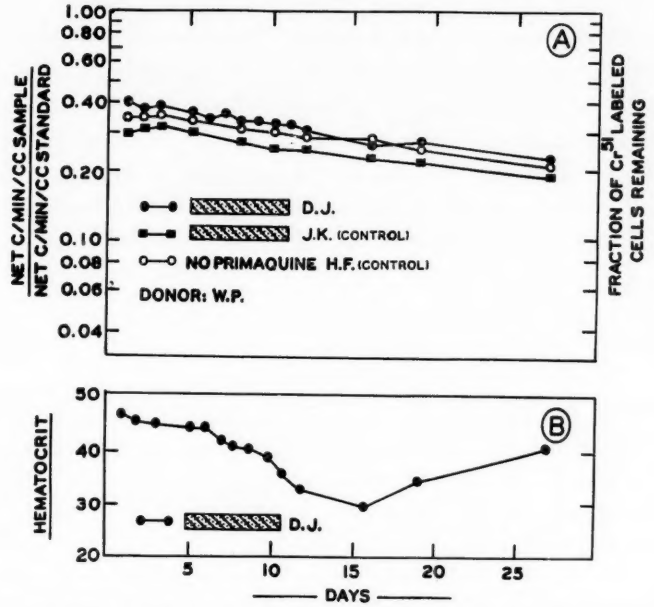

Fig. 4.—The fate of red blood cells from a normal (nonsensitive) man when transfused into a primaquine-sensitive volunteer who was then given primaquine, and into suitable controls. Solid symbols: primaquine, 30 mg. base daily for the period indicated by the hatched block. Hollow symbols: no drug. J. K.: Caucasian recipient. D. J. and H. F.: primaquine-sensitive Negro recipients. Donor W. P.: normal (nonsensitive Negro).

A, Red cell survival curves. *B*, Hematocrit determinations during hemolytic episode induced by primaquine in D. J.

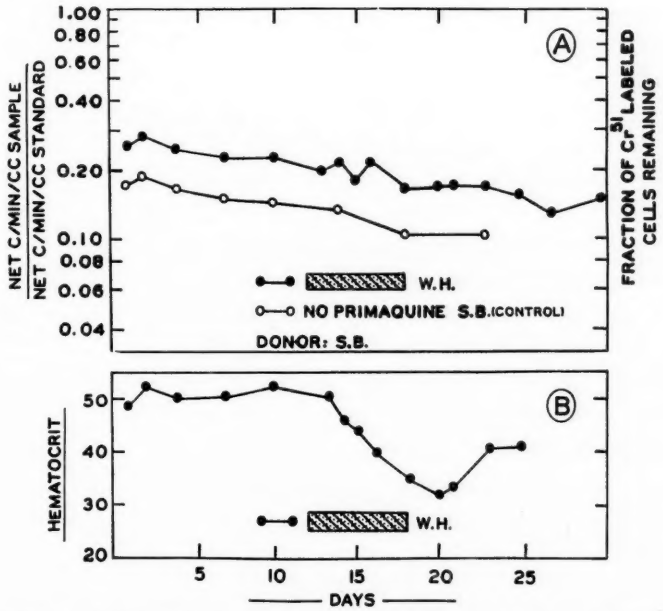

Fig. 5.—The fate of red blood cells from a normal (nonsensitive) man when transfused into a primaquine-sensitive volunteer who was then given primaquine, and into suitable controls. Solid symbols: primaquine, 30 mg. base daily for the period indicated by the hatched block. Hollow symbols: no drug. W. H.: primaquine-sensitive Negro recipient. S. B.: normal Negro recipient. Donor S. B.: normal (nonsensitive) Negro.

A, Red cell survival curves. *B*, Hematocrit determinations during hemolytic episode induced by primaquine in W. H.

Group II. Cells From Normal Men in Primaquine-Sensitive Recipients.—The results of the converse experiments, wherein labeled red blood cells from normal men were transfused into primaquine-sensitive Negroes and suitable controls, are illustrated in Figs. 4 and 5. There was no hemolysis of the transfused normal cells when primaquine was given (Figs. 4, *A* and 5, *A*), although the recipient developed severe anemia due to the hemolysis of his own cells (Figs. 4, *B* and 5, *B*). None of the controls exhibited a change in hemoglobin concentration or hematocrit throughout the experiment. The control survival curves of cells from normal men transfused into normal men (given primaquine) and into primaquine-sensitive Negroes (not given primaquine) were essentially identical.

The pattern of the survival curves of the labeled cells was similar to the controls during both the hemolytic and recovery phase of the recipient. A slight hemolysis of the normal cells might be masked by a shrinkage of the recipient's blood volume during the hemolysis, but any destruction of the labeled cells would have become evident during the recovery phase. The data indicate that cells from normal subjects transfused into sensitive recipients were not affected by primaquine administration and that the recipient's blood volume did not change during the experiment.

The Coombs' test was negative before and during the experiment. At no time could sickling of the cells from the sensitive men be demonstrated. Osmotic fragility tests were normal before and during the experiments on all men.

SUMMARY

A study was made of the acute hemolytic anemia which occurs in a small group of members of heavily pigmented races on administration of the antimalarial drug primaquine (an 8-aminoquinoline). The localization of this defect to intra- or extracorpuscular causes was investigated in a group of susceptible American Negroes.

Red cells from primaquine-sensitive men, when transfused into normal (primaquine nonsensitive) men, were partially lysed on the administration of the drug to the recipients of the cells. Previous splenectomy of a recipient did not affect the rate of hemolysis.

Red cells from normal men were not lysed when transfused into primaquine-sensitive Negroes who were receiving the drug, although the recipients developed an acute hemolysis of their own cells.

Attempts to produce sickling of the red cells of primaquine-sensitive men were not successful and the Coombs' test was negative throughout the induced hemolytic episodes.

CONCLUSION

The drug-induced acute hemolytic reaction occurring in primaquine-sensitive subjects is due to a unique susceptibility of the red blood cell rather than to abnormal extracorpuscular factors developed in response to the administration of primaquine.

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LABORATORY METHODS

AN INSTRUMENT AND METHOD FOR RAPID, DEPENDABLE DETERMINATION OF FREEZING-POINT DEPRESSION

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INTRODUCTION

THE instrument and method described were developed to enable relatively inexperienced personnel to do accurate cryoscopic osmotic activity determinations of 1 ml. samples of aqueous solutions rapidly and dependably to better than ±2 per cent or one milliosmol per liter, whichever is greater, over the range of 0 to 1.85 "osmolal"* concentrations.

Thermistor thermometry has been utilized in many modifications[1, 2] for this problem, but it is felt the present system provides the most satisfactory, practical, and inexpensive solution.

Refrigeration methods have, in general, utilized liquid baths and depended on evaporation of solvents, solid CO_2, or ice mixtures, which require considerable attention to provide reproducible conditions for cooling. We have adapted the Ranque-Hilsch vortex tube[3, 4] to provide a simple refrigeration system that requires no baths and gives reproducible rates of cooling requiring only a regulated laboratory compressed air supply of moderate capacity.

In our method, there is no attempt to make precision measurements of absolute temperature, but, rather, the end point is always compared to readings of standard solutions and the actual value is obtained by interpolation. By this system, only moderate demands are made on the stability of the thermistor and thermistor bridge, and these may be easily met.

The freezing unit consists of a multi-walled chamber containing air near freezing temperature, thus giving adequate isolation and control to keep a reasonably constant temperature. A constant-speed motor to provide stirring and a probe-positioning device are also utilized to further reduce variations in the temperature gradient.

The complete instrument is composed of two parts: the thermistor bridge null detector unit and the freezing unit.

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*A standard of one "osmolal" concentration has a freezing-point depression of 1.86° C., or has the concentration of one mole of "ideal" solute per 1,000 Gm. of H_2O.

MATERIALS AND METHODS

Detecting Unit.—Basically, the thermistor temperature indicator consists of a thermistor probe, an A.C. bridge with capacity compensation, and an amplifier-type null detector indicating balance by maximum shadow angle on an electron ray indicator tube.

The thermistor probe consists of a bead of temperature-sensitive material, the associated holder and leads. The bead as purchased is sealed in glass and may be considered permanent unless the glass is broken. It is a type 14-A bead made by Western Electric with a resistance of approximately 355,000 ohms at 0° C. The probe is constructed by sealing the bead assembly into the end of a 10 cm. length of 4 mm. glass tubing to support it and protect the leads. The glass probe is filled with semisolid silicone grease to obviate the problem of moistureproof seals capable of standing the freezing-thawing cycle. It is of utmost importance that the leads be protected against moisture condensation inside the portion that is cooled since the bridge is capable of showing unbalance due to a change of resistance of only a few parts in a hundred thousand and a moisture film will represent a considerable shunting effect across the high resistance thermistor. The leads to the probe are arranged to have minimum capacity shunting the thermistor by shielding each one individually.

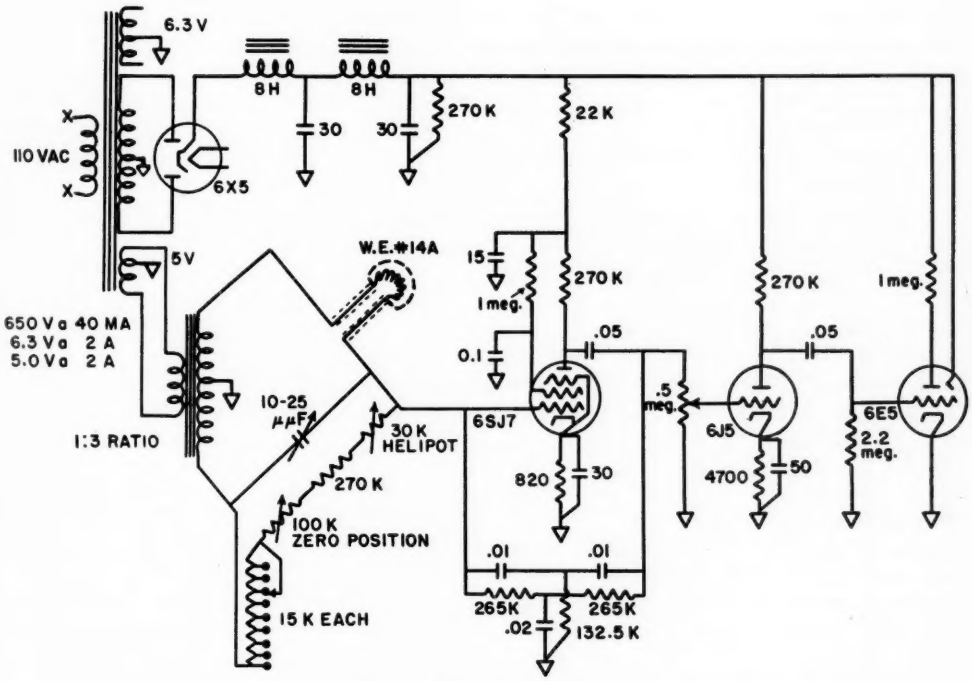

Fig. 1.—Circuit diagram.

The bridge (Fig. 1) consists of a center-tapped interstage transformer, energized from the 5 volt winding of the power transformer, supplying approximately 16 volts across a range resistor, step switch, and helical potentiometer on one side, and the thermistor on the other. The center tap of the transformer is grounded and the point between the two arms is fed to the input of the null detector. A ceramic trimmer capacitor is provided to compensate for cable capacity phase shift, thereby sharpening the null. Sensitivity is controlled by means of the potentiometer in the 6J5 grid circuit.

The high gain narrow band amplifier is designed to reject any harmonics found in the bridge transformer that tend to broaden the null. The amplifier is tuned to within one

cycle of 60 cycles per second and will perform at maximum gain only when used on true 60 cycle power line. A departure of only 5 cycles per second will reduce the gain of the detector to one-half. This must be kept in mind wherever the possibility of a power line frequency variation exists. This condition, however, would exist only where power is obtained from local motor generator sets.

A temperature change of 1° C. causes a resistance change in the thermistor of 4.7 per cent of its value or 16,700 ohms at 0° C. The step switch and the helical potentiometer provide the coarse and fine measuring elements to balance these resistance changes. Each step of the step switch is 15,000 ohms or about 0.1° C. (This overlapping enables one to select a convenient position of the Helipot for standards and unknown.) The detector is capable of sharp detection of one dial division of the helical potentiometer which is equivalent to 30 ohms or 0.0018° C. This corresponds to the freezing-point depression of 1 milliosmol in aqueous solutions.

Determination of freezing-point depression by this method depends upon providing reproducible temperature gradients, mixing, degree of supercooling and induction of freezing. No attempt is made to obtain actual temperature values, but the sample is compared with standards of known osmolarity and so closely bracketed that interpolation is not open to question. The values obtained are reproducible within the limits of tolerable variability over a period of a few hours so that standards need only be repeated every few hours to maintain accuracy. The foregoing statement requires only that room temperature is moderate and does not fluctuate rapidly.

The thermistor does not have any specific resistance, just so it reads zero centigrade on one of the higher steps on the step switch. The step switch resistors are only approximate and provide a reproducible scale selection and a means of indicating when to seed for induction of freezing.

Freezing Unit.—The freezing unit consists essentially of a Lucite multi-walled cooling chamber, a stirring system, and a thermistor guide. The stirring system and thermistor guide are features which allow easily reproducible conditions.

The freezing chamber itself (Fig. 2) is composed of a number of Lucite elements fitted together by friction joints (not cemented) so that the chamber may be conveniently disassembled. The basic element is a modification of the Ranque vortex tube, machined from a solid Lucite rod. The tangenitally placed entrance jet (a) causes the air in the vortex chamber (b) to spin at high speed, expanding the central air and compressing the peripheral air. The cold air is forced out the central small exit (c) by increasing the central region pressure above ambient by a throttle distal to the hot exit tube (d). To reduce noise, a muffler may be placed beyond the throttle. A tube stop (e) holds the sample tube in place while allowing the cold air to circulate about it. Surrounding the test tube is a double-walled cylinder (f), completely closed except for four small holes communicating with the tube space and acting as an outlet for the cool air. The outer wall of this cylinder serves as the inner, stationary portion of a Nylon ball bearing. The outer, the revolving part of the ball bearing, is a third concentric wall (g). Grommets (h) fitted into this wall, hold the sample tube firmly. Ordinarily 13 by 100 mm. test tubes are used. Air pressure of 35 pounds (a flow of about 3.3 cu. ft. free air per minute) is sufficient for freezing samples in about 1½ minutes. This is less than the amount of air ordinarily supplied adequately by a ½ HP. air compressor.

The stirring method, which makes possible smooth, constant mixing, utilizes a motor which rotates the ball bearing by friction drive at about 200 r.p.m. This in effect turns the sample tube since the tube holder is an extension of the rotating part of the ball bearing.

The thermistor guide consists of a vertical rod to which the probe is attached. A groove-and-stop arrangement enables the thermistor to be lowered into the sample tube at a reproducible position.

Standards.—The standards used were at concentration intervals no more than 100 milliosmols per liter apart. For testing purposes, standards of NaCl from 0 to 1 molar con-

centrations at 0.05 molar intervals were made up, as they are easily prepared. The standard concentration values were corrected to give molal concentrations using the Handbook of Chemistry and Physics[5] and these corrected to the corresponding "osmolal" (footnote on title page) concentrations, using data from the freezing-point depression tables in the International Critical Tables.[6] By so doing, the unknown may be determined and expressed as the fraction of "ideal" moles per kilogram of water.

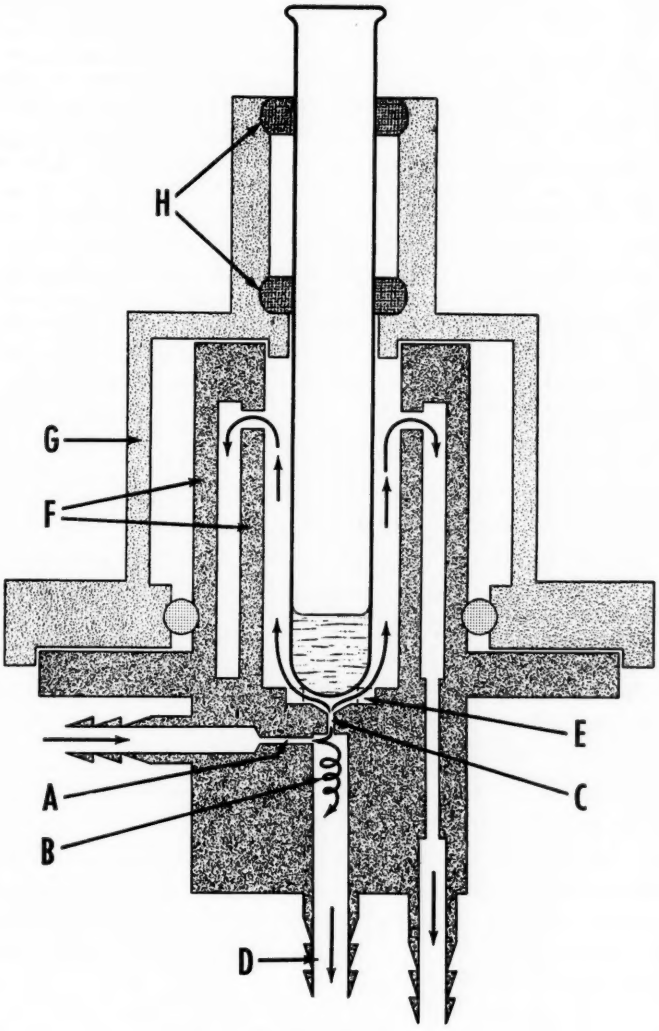

Fig. 2.—Freezing chamber.

Method.—Both parts of the apparatus should be well grounded.

Samples should start from approximately the same temperature in order to get the best results.

To make a determination, the probe is placed in the sample tube containing 1 ml. of standard or unknown. The stirring motor is turned on and the stationary thermistor eccentrically placed serves as a stirring rod. The thermistor guide and tube stop assure that

the probe and tube will always be in the same relative position. A "scout run" is made to determine the general range of the unknown. The step switch should be on the highest position and the sensitivity knob set to the point at which the "eye" is just beginning to open. The air pressure is turned on. As the sample cools and approaches the temperature indicated by the highest step, the "eye" will open to maximum shadow angle, and will begin to close as the temperature is passed. At this point the step switch is turned down one step. As the "eye" opens to maximum again we switch down another step. This is repeated at each maximum opening until the sample freezes spontaneously (which may be indicated by a sudden change in the "eye" opening, indicating the rapid temperature change). At this point the air is turned off, the sensitivity turned up about two-thirds of maximum and the step switch rotated until the widest "eye" opening is observed. The Helipot dial is then rotated until full opening is observed. The sensitivity is increased to maximum and balance maintained with the Helipot until a plateau is reached. This is recognized as the point at which the full shadow angle is stable for a period of five to twenty seconds, depending on the concentration of the sample. This point is the approximate reading for the sample. For an exact reading, a fresh sample (or the previous sample completely thawed) is cooled in the same manner but is seeded by AgI* on a wooden applicator stick when balance occurs with the switch set two steps below the scout reading. (This is approximately 2° supercooling.) Cooling is then turned off, the step switch returned two steps and balance maintained as before until the plateau is recognized. This Helipot setting is the reading taken for the sample. As the step switch is not a precision element, the unknown and standards must be read on the same step switch position to be compared, and linear interpolation is used to find the unknown osmolarity in terms of the standards.

RESULTS

From three to six readings were taken on each of the standards of NaCl, prepared as described in the paragraph on standards. The repeat readings on any given standard were taken over periods of two and one-half to three hours. In concentrations greater than 0.100 osmol per liter the maximum deviation from the average reading would give an error of 0.5 per cent in terms of the actual value of that standard. Using the average readings obtained and computing the value of each by interpolation between the bracketing standards, the greatest error in standards of 0.100 osmol per liter and higher concentrations was 1.2 per cent.

A number of blood plasmas and urines were determined by the same method. These ranged in concentration from 0.140 to 0.409 osmol per liter. Repeatability was within two Helipot divisions (3 milliosmols) and that the cooling, seeding, and plateaus observed followed the same pattern as those of the standards makes it seem reasonable to assume that the answers obtained have the same accuracy as was attained when making determinations on NaCl standards.

SUMMARY

A method has been described for determining the freezing point of aqueous solutions featuring simple, low-cost apparatus, ease of manipulation, rapidity, and accuracy. The simple freezing method utilizes a stream of chilled air from a Ranque-Hilsch vortex tube for freezing the specimen and a thermistor probe-bridge assembly for indicating the end point.

*AgI was chosen as its crystal form is epitaxial to ice so that it will initiate ice crystal formation. The solubility is too low to influence the results.

The thermistor bridge utilizes a Western Electric 14-A thermistor and a simple tuned amplifier with an electron eye null indicator. The dial of a ten-turn helical potentiometer reads 0.0018° C. per division. Standards can be read to one division and are stable to one or two divisions over adequate time to record a few unknowns. Unknowns, alternated with standards every few determinations, are interpolated to give the apparent osmolarity to within 2 per cent.

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A RAPID METHOD FOR THE DETERMINATION OF B_{12} BINDING POWER IN THE GASTRIC JUICE

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IN A recent communication[1] from our laboratory it was reported that vitamin B_{12} can combine with a substance in gastric juice in such a manner that the resting cells of *Lactobacillus leichmannii* are unable to adsorb it. This observation enables us now to develop a simple procedure for the quantitative determination of the binding power for vitamin B_{12} in gastric juice derived from clinically healthy individuals of different age groups or from patients with various diseases. The details of the procedure and the results of the determinations are reported in this communication.

PRINCIPLE

Free vitamin B_{12} can be quantitatively adsorbed to resting cells of *L. leichmannii*, and therefore can be removed from a solution by centrifugation. The addition of gastric juice to vitamin B_{12} results in the formation of a complex from which vitamin B_{12} cannot be removed by adsorption to the microorganisms. The amounts of Vitamin B_{12} in millimicrograms and of a gastric juice in milligrams of nitrogen bear a quantitative relationship which provide the basis of our method to be described below.

EXPERIMENTAL DETAILS

Gastric juice was obtained from a fasting individual, without either histamine or alcohol stimulation. After centrifugation to remove any sediment, the pH of the specimen was determined with a glass electrode. A measured volume (usually 5 ml.) of the sample was neutralized to about pH = 7 (6.8-7.2) with a small amount of NaOH of such a concentration (0.1 N to 1.0 N) pending on the acidity of the sample, as to result only in a slight increase in the total volume. An aliquot of the neutralized gastric juice was used for the colorimetric microdetermination of total nitrogen. After digestion with concentrated H_2SO_4, the color was developed with the Nessler's reagent and compared with a standard. The remaining portion was saved for the determination of binding power as follows: One-half ml. of the test solution was added to tube No. 1 containing 1.5 ml. of physiological saline solution. Three serial twofold dilutions were prepared using 1.0 ml. of solution No. 1. One ml. of the content in the fourth or last tube was measured and discarded so that all four tubes now contained 1 ml. of different dilutions of the test substance. To each of these tubes were then added 2 ml. of saline solution and 1 ml. of a standard radioactive vitamin B_{12} solution (20 millimicrograms per milliliter). After standing at room temperature for 20 minutes, 1 ml. of resting cells suspension of *L. leichmannii* prepared according to the directions of Davis and Chow[2] was added to all tubes. The mixtures were allowed to stand at room temperature for 30 minutes and centrifuged at 2,000 r.p.m. for 20 minutes. The supernatant fluid was removed by suction with a fine capillary. The packed cells were transferred quantitatively to a planchet with water and dried. The radioactivity adsorbed to the microorganisms was then counted with a thin window Geiger counter. The obtained count is a measure of the fraction of vitamin B_{12}

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not bound by the gastric juice in the dilution tested. To determine the radioactivity adsorbed to the cells in the absence of gastric juice, 1 ml. of the standard vitamin B_{12} solution was added to 3 ml. of the saline solution without any gastric juice (in tube 5). After standing at room temperature and centrifugation as described above, the radioactivity adsorbed to the microorganisms was estimated and it represents the total free B_{12}. The amount of vitamin B_{12} bound to the gastric juice can then be estimated from the differences between the radioactivity of the control (No. 5) and each of the experimental ones containing varying amounts of gastric juice. An alternate procedure for the determinations of free B_{12} was adopted when certain preparations of the resting cells suspension was unable to adsorb quantitatively the added free vitamin B_{12}. One ml. of the resting cells suspension was added to a series of five tubes containing 0.0, 5.0, 10.0, 15.0, and 20.0 millimicrograms of radiovitamin B_{12} in 3 ml. of 0.85 per cent NaCl solution. The radioactivity adsorbed to the cells was measured after centrifugation and transferring to the planchet. A standard curve was constructed by plotting the adsorbed radioactivity against the added vitamin B_{12}. From this curve the amounts of free vitamin B_{12} in the test solutions were estimated.

RESULTS

The Quantitative Relationship Between Vitamin B_{12} and Gastric Juice.— The results given in Table I indicate that within the range of our experimental dilutions the ratio of vitamin B_{12} bound to the gastric juice expressed as micrograms of B_{12} bound per milligram of nitrogen in gastric juice is essentially constant for any one sample. This constancy is independent of the ratio of the concentrations of the two reactants and can be taken as a measure of B_{12} binding power. This phenomenon is true regardless of the potency of the test fluids. To test the reproducibility of our results aliquots of a neutralized specimen obtained from a healthy subject were kept in a freezing cabinet in sealed ampules. Whenever an assay was to be made the contents of one vial were carefully thawed and assayed. The results obtained over a four to five months' period are tabulated in Table II. They show the variations that can be expected in our routine assays. In this series three samples of radioactive B_{12} with specific activities ranging from 40 to 120 microcuries per milligram* were used.

TABLE I. RATIO OF B_{12} (MILLIMICROGRAMS) BOUND TO GASTRIC JUICE (MILLIGRAMS N) WITH DIFFERENT BINDING CAPACITY

SAMPLE	MMCG. B_{12} BOUND/DILUTION	MG. N/DILUTION	MMCG. B_{12} BOUND/MG. N
A	5.4	.124	43
	3.3	.062	53
	1.2	.031	49
B	16.6	.078	212
	8.5	.039	218
	5.0	.020	250
C	18.4	.231	80
	9.5	.116	82
	5.2	.058	90

Usually only three out of four test dilutions will fall within the limits of our standard curve.

*We are indebted to Dr. C. Rosenblum of Merck and Company for his generous supply of radioactive vitamin B_{12} which was used in this study with the permission from the Isotope Division of the Atomic Energy Commission.

TABLE II. VARIATIONS IN GASTRIC JUICE ANALYSIS FOR COMBINING POWER

CALENDAR DATE	MCG. B_{12}/MG. N
9-18	[illegible]
9-26	77
10-17	76
10-21	84
11-15	80
12-1	92
1-12	90
1-27	82
2-2	86
	82 ± 6.3*

*Average ± standard deviation.

Binding Power of Gastric Juice From Various Individuals.—The binding power in the gastric juice of subjects with pernicious anemia and "old" individuals (75 years or older) was measured. Samples from clinically healthy mature adults were used for comparison. The results of such a study are given in Tables III and IV. It can be seen that the gastric juice samples from the controls combine with vitamin B_{12} to the extent of 71 millimicrograms per milligram of nitrogen with a standard deviation of the mean of ± 3. The corresponding figure for specimens obtained from adult pernicious anemia patients (Table III) is only about 16 millimicrograms. The difference is statistically significant.

TABLE III. INTERRELATIONSHIP OF DISEASE AND B_{12} BINDING

DISEASE	B_{12} BINDING POWER*
Control	71 ± 3 (10)†
Pernicious anemia	16 ± 4 (7)
Juvenile pernicious anemia	80 ± 5 (3)
Ulcers	48 ± 18 (4)

*Millimicrogram B_{12} bound to each mg. of nitrogen in gastric juice.
†Numbers in parentheses indicate the number of subjects analyzed.

TABLE IV. INTERRELATIONSHIP OF AGE AND B_{12} BINDING

AGE (RANGE)	pH		
	0.7-2.0	2.5-5.0	5.0-7.0
57-85	50* ± 3.6 (11)†	126 ± 7 (10)	104 ± 13 (17)
25-45	71 ± 3 (10)	—	—
10 or less	—	104 ± 20 (4)	—

*Expressed as millimicrograms B_{12} bound to each milligram of nitrogen in gastric juice.
†Numbers in parentheses indicate number of specimens analyzed.

It is interesting to note that samples from the patient with so-called juvenile pernicious anemia* showed much higher B_{12} binding power. Although in patients with ulcers this measure of B_{12} binding capacity was low, on the average, variation between samples was too great to permit any broad statements of significance to be made. When the data on binding power of gastric juice samples from old donors are divided into two groups, depending

*We wish to express our sincere appreciation to Dr. D. Watkin of the Section on Gerontology of the National Institutes of Health, Dr. L. Finberg of the Baltimore City Hospitals, Dr. E. Reisner of the New York Hospital, Dr. J. Krevans and Dr. L. Conley of the Johns Hopkins Hospital and Dr. G. Rake of the Squibb Institute for Medical Research for the collection of gastric juice specimens.

on the presence or absence of achlorhydria, the mean value for the low pH samples is lower than the control mean; that for the high pH samples higher. These results are reported to indicate the existence of a possible correlation between pH and binding power in gastric juice, although the physiological interpretation of such data must await further experimentation.

DISCUSSION

The binding power of a substance for B_{12} can be measured in a number of ways. According to the technique of Ternberg and Eakin,[3] the test substance in varying concentrations is added to a known amount of vitamin B_{12}. The entire mixture is then assayed for the microbial activity of this vitamin with the usual procedure. The decrease in B_{12} activity is taken as a measure of the binding power. It is obvious from this type of experiment that inhibitors as well as binding substances can produce the same results. Later, Bird and his associates[4] found that bound B_{12} was nondialyzable. The amount of the test substance necessary to bind a given quantity of B_{12} provides another basis for the measurement of binding power. The experiments described in this communication take advantage of the fact that B_{12} bound to gastric juice is not adsorbed by resting cells of *L. leichmannii*. Since these three methods are based on entirely different principles, it is hardly to be expected that they will necessarily give the same quantitative or even qualitative results.

Since gastric juice can bind vitamin B_{12}, it is interesting to speculate on the significance of this property to the physiological action of intrinsic factor.[5] Its importance has been minimized because of the reported finding of chemical substances[1, 6] capable of binding B_{12} but not of potentiating oral B_{12} therapy. In a recent paper, Latner and his associates reported the fractionation of gastric juice. Binding power could be demonstrated in more than one fraction but only one contained intrinsic factor properties. These experiments taken as a whole, therefore, suggest that while substances capable of binding with vitamin B_{12} need not possess the intrinsic factor properties, nevertheless, the intrinsic factor in the gastric juice or even hog duodenum might combine with vitamin B_{12}. The proof of this hypothesis will come only after the intrinsic factor is isolated in a pure state according to the rigid chemical and physical tests. Therefore, any quantitative measurement of the binding power of gastric juice is of interest.

This communication reports a simple and unique procedure for the measurement of the binding power of gastric juice from a variety of subjects; a property which may be related to the intrinsic factor activity. This procedure may also be useful in the fractionation of binding substance in gastric juice. Our data demonstrate that the binding power of the gastric juice of young subjects is significantly higher or lower than in specimens of old individuals, according to the absence or presence of achlorhydria, respectively. The acidity of the gastric juice must play an important role on the stability of the binding substance. Hence, samples from different age groups were divided according to their pH and then compared. If the data on the old indi-

viduals can be substantiated by further studies it will then be easy to ascribe the low tissue reserve for B_{12} of the aged,[8] to the lack of binding power in the gastric juice. Verification and extension of these hypotheses must await the accumulation of more experimental data. However, it must be pointed out that our determinations provide informations only on the concentration but not on the total available binding substances in the gastric juice which takes into account both concentration and volume. The latter was not measured in the present study.

SUMMARY AND CONCLUSION

Vitamin B_{12} combines with one or more substances in gastric juice in such a manner as to make it unadsorbable by resting cells of *L. leichmannii*. This fact enables the development of a method for the measurement of the binding power of the gastric juice. With this procedure it was demonstrated that specimens obtained from pernicious anemia patients and from old individuals without achlorhydria had significantly lower binding power than control specimens. Gastric juice specimens from three cases of juvenile pernicious anemia and from old people with achlorhydria contained at least as much as the normal samples. The importance of such findings is discussed.

We wish to acknowledge the grant-in-aid from the Atomic Energy Commission.

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THE DEMONSTRATION OF FAT EMBOLI IN TISSUE SECTIONS USING PHOSPHIN 3R, A WATER-SOLUBLE FLUOROCHROME

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POPPER, investigating the distribution of vitamin A in tissues and organs, used phosphin 3R to stain the lipoid material in the tissue sections.[6] While developing a technique for detecting fat emboli in the circulating blood using this stain, it was only natural to apply it in demonstrating fat emboli in tissue sections.[5] It has proved to be quite satisfactory.

METHOD

Material.—Fresh tissue or tissue fixed with 10 per cent formalin.

Solutions.—Phosphin 3R,* 1 = 1,000 aqueous solution.

Procedure.—Cut frozen sections. Rinse lightly in distilled water. Stain in Phosphin 3R solution for 5 minutes. Rinse lightly again in distilled water. Use as wet mounts, or mount with any nonfluorescent mounting medium.

Sections should be examined under fluorescent light using the ordinary equipment of fluorescence microscopy. The fat emboli fluoresce to brilliant white.

RESULTS

In Figs. 1 and 2 are shown examples of fat emboli in man and dog as demonstrated by the above procedure. Fig. 1 shows fat emboli in the pulmonary arteries (arrows) of an aged patient dying after multiple fractures. Fig. 2 shows fat emboli in the pulmonary arteries (arrow) of a dog sacrificed after multiple fractures.

DISCUSSION

The technique of fluorescence microscopy utilizing the natural fluorescence of biological materials and fluorochrome stains has been well described.[2, 3, 4, 7] This method has been used to study oils causing lipoid pneumonia[11] and aspiration pneumonia.[10] Phosphin 3R belongs to the xanthine group of stains.[1] It has been used to demonstrate tubercle bacilli droplets[8] of neutral fat in the urine and the feces[9] and fat emboli in the blood.[5]

The chief advantages of this method are its simplicity, only one stain or solution is required, and the absence of contact with fat solvents during staining. This latter feature should preserve the fat in the tissue section inviolate, a point of some importance when only a small number of fine fat emboli are present.

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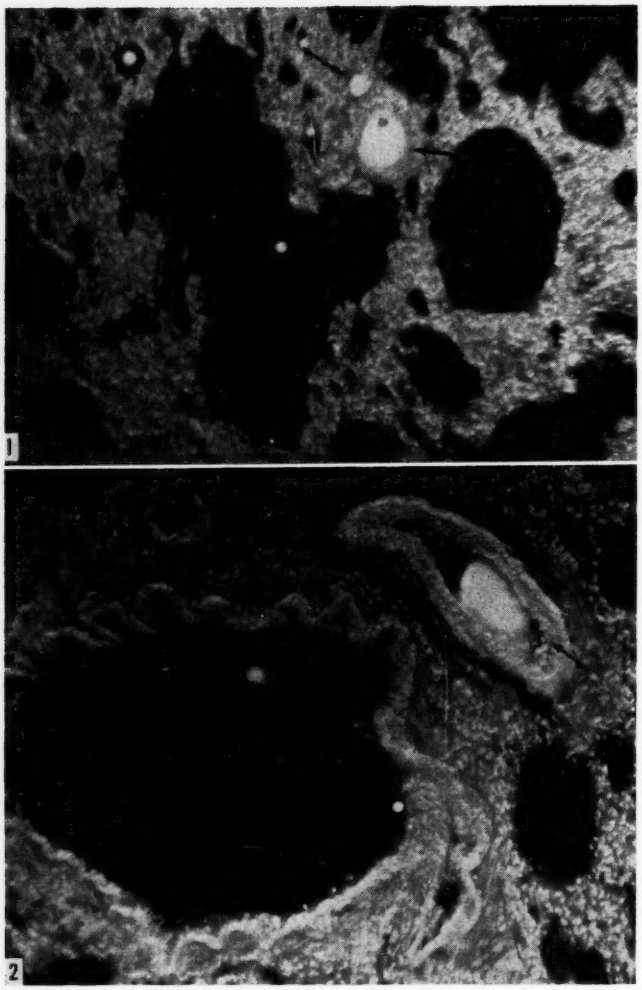

Figs. 1 and 2.—Photomicrographs using fluorescence microscopy demonstrating the appearance of fat emboli in the lung. The stained fat has a brilliant white fluorescence. Fig. 1, Pulmonary fat emboli in an aged patient dying after multiple fractures. Fig. 2, Pulmonary fat emboli in a dog sacrificed after multiple fractures.

CONCLUSION

A method of staining fat emboli in tissue sections using Phosphin 3R, a water-soluble fluorochrome, has been described. The advantages of this technique are its simplicity, and the absence of fat solvents in the solutions used.

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A CONTINUOUS INTRAVENOUS INFUSION APPARATUS FOR THE UNANESTHETIZED DOG

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DURING the course of certain studies it became essential to infuse unanesthetized dogs over extended periods while allowing them freedom of activity within a cage. The apparatus here described (Fig. 1) fulfilled these requirements. The simplicity of construction and operation through incorporation of easily available commercial parts offered advantages over previously described infusion apparatus (Jacobs, 1931).

The infusions were carried out in a 24″ × 28″ × 32″ animal metabolism cage having coarse steel screening on the top and sides. Affixed to the center of the top panel is a self-aligning ball-bearing flanged cartridge (Boston BNFS ¾″). This is bolted in place to a ⅛″ brass securing plate having a 1″ center hole. This bearing acts as a swivel for the 36″ cable (½″ BX Armored Cable or GE Flexible Conduit). A 2″ sleeve (½″ GE Metallic Tubing), sweated over one end of the cable, is secured in the flange bearing collar with set screws. The opposite end of the cable is securely clamped into a connector (GE SP7051 Reversible Connector) which is anchored to the collar of the dog. The cable allows complete freedom of motion within the cage and protects the Tygon tubing. Attachment to the dog is by means of a common commercial dog harness. The collar was fashioned from a ¼″ × 2″ × 15″ strap of stiff horsehide to the center of which was riveted a metal plate (1¾″ × 4″ GE outlet box cover blank with a single knockout). The cover plate and collar were shaped to conform to the contour of a dog's neck. The reversible connector was attached to the collar with the lock nut provided. Two adjustable leather straps secured the collar on the dog's neck. The collar was secured to the harness with thongs. Additional web straps, connecting the chest and bellybands inside the dog's legs, prevented twisting of the harness. A rubber stopper was fitted over the hub of a spinal needle (Quincke 20G or Flexible 19G 3½″). The flexible needle shaft was bent sharply at its junction with the hub and shaped to the circular form of the collar. The needle shaft was then cut off square 2 inches from the hub and the stylet withdrawn. A cloth-faced sponge rubber pad prevented chafing. Tygon 3/16″ tubing 40″ long was run down through the cable and attached to the adapter. The other end was fitted to the freely moving sleeve arm of a glass blower's swivel.† This swivel moved freely and was leakproof at the

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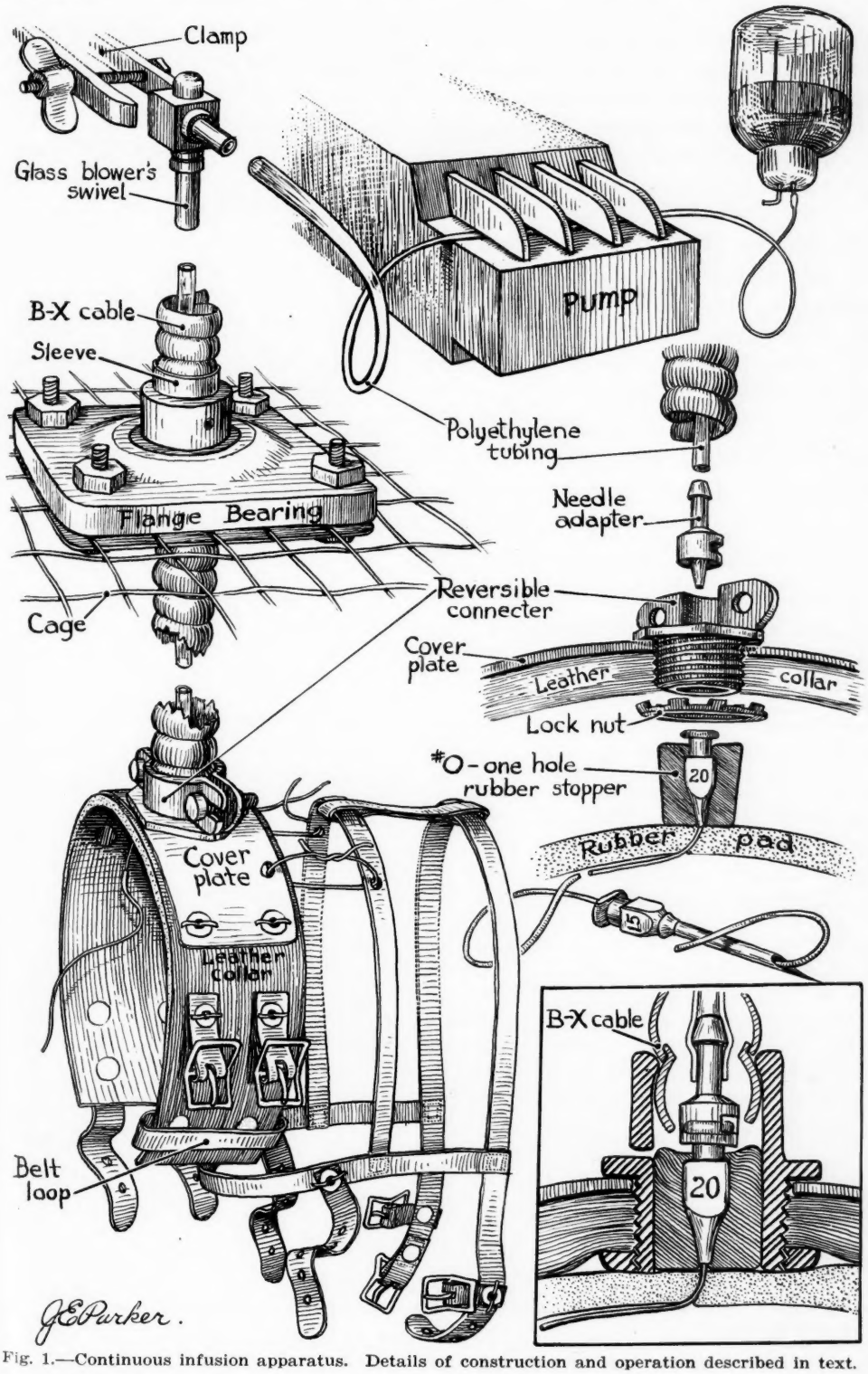

Fig. 1.—Continuous infusion apparatus. Details of construction and operation described in text.

pressures obtaining during infusions of 0.50-8.0 ml./min. However, as a precaution, a single turn of soft fine cotton twine was wound in the space between the rotating sleeve and standing shaft cuff. The swivel was fixed in a clamp directly over the flange bearing with the rotating sleeve pendant. The horizontal arm of the swivel was connected with a 36″ length of 3/16″ pure gum tubing through the Bowman Infusion Pump (Model 2)* to the reservoir.

The fluid to be infused is pumped through the tubing system to wash out any air. The harness is then secured on the dog. With the dog lying on its side a short-bevel 1½″ 15G needle is inserted in the jugular vein in the mid-neck. An 8 inch length of polyethylene tubing (PE90; 0.86 × 1.27 mm.) is threaded through the needle into the vein. The needle is withdrawn over the tubing and the free end of the tubing is connected with the spinal needle in the collar. The infusion pump is started immediately. A strip of adhesive tape, banded around the collar and needle, provides fixation for the needle. Finally, the collar is strapped on and fastened to the harness.

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*Process & Instruments, Brooklyn, N. Y.

A PRACTICAL ACCURATE METHOD FOR MEASURING AND RECORDING THE ACTIVITY OF PROTEOLYTIC ENZYMES

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INTRODUCTION

NUMEROUS investigators have devised methods to measure the activity of proteolytic enzymes. Most of these methods involve chemical or physical analysis,[1-6] although some have used photometric analysis,[7-14] and electrical conductivity.[15-17] We have developed in this laboratory a method for the measurement of proteolytic enzyme activity that will record the extent of this activity at ten-second intervals on a time axis. This device is a modification of the blood coagulation-time apparatus of Richardson and Bishop.[18] It features a simple electrical circuit and a permanent recording with considerable fidelity.

METHOD

The apparatus used is composed of a 110 v a. c. motor attached to a gear that oscillates back and forth through 90° in such fashion that an attached platform will swing 45° up and down from horizontal. A full oscillation occurs every ten seconds. On the platform attached to the shaft of the gear is placed a container for the substrate, and a mercury switch. This switch is in reverse position to the electrodes in the end of the container, so that the circuit is complete only when the substrate alone, and not the enzyme solution present, affects the conductivity of the series circuit. With this arrangement, when gelled proteins such as albumin, gelatin, blood, and the like are in contact with the probes, solutions containing proteolytic enzymes placed in the enclosure progressively hydrolyze and decrease the volume of the substrate and thereby diminish the conductivity of the system with each oscillation. A diagram of the apparatus is shown in Fig. 1, which consists of a d. c. source such as a battery or 6 v battery-eliminator, a 5 Ma., 70 ohm Esterling-Angus ink-writing recorder, and a 500 ohm resistor in series with the instrument to protect its movement. Fig. 2 shows the complete apparatus as operated.

Easily obtainable polystyrene plastic tubes with polyethylene friction caps have been used successfully as containers. The tubes, 1.5 cm. × 5 cm., have been modified by adding conductive electrodes fixed solidly in the flat base, 8 mm. apart. We have found 0.2 ml. of substrate to be a practical volume for reliable measurement. The protein is introduced into the tube while vertical, in sol form and allowed to gel, to ensure a standard geometric form. After the gel has formed, the proteolytic enzyme may be introduced, and oscillation of the apparatus started. Full hydrolysis occurs typically in five to thirty minutes, depending on the concentrations of the materials used.

RESULTS

Fig. 3 shows a typical recording using 0.2 ml. of 5 per cent gelatin as substrate, and 0.1 mg. of buffered trypsin* in 1.0 ml. of solution as the proteolytic enzyme. Activity may be determined as time for a given fractionate volume of

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digestion, or as the substrate decay per unit of time, both standard procedures in analyses of enzyme activity. Conductivity is an almost, but not perfect, linear function of volume of substrate (Fig. 4), but the small error over the curve can

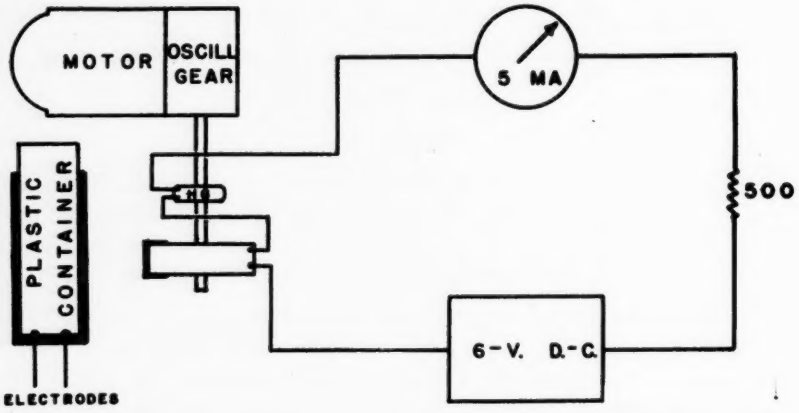

Fig. 1.—Circuit diagram of the apparatus used for proteolytic enzyme studies.

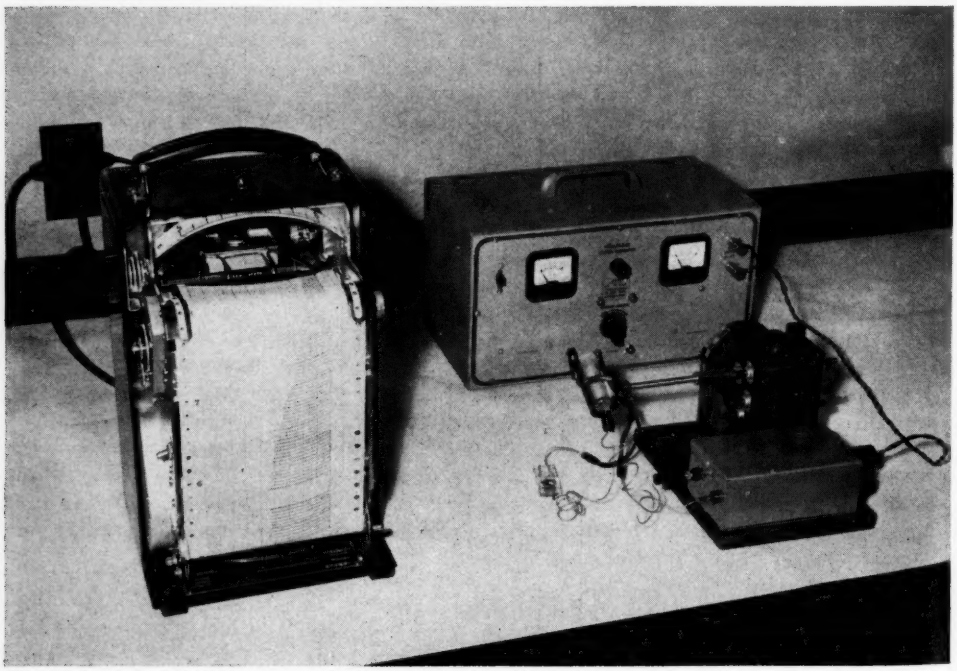

Fig. 2.— Photograph of complete apparatus.

be corrected by calibration. This method offers a feature of having a constant 2-dimensional area of substrate presented to the enzyme with each oscillation, not possible with many methods presenting 3-dimensional surfaces for activity,

but the loss of substrate into the enzyme medium presents the problems of mass action inherent with other methods. The first half of the curve is more accurate and reliable than the second half, because at termination contact may be broken just prior to complete digestion. We have found the first half of the curve exceedingly reliable. Other substrates such as lipids and waxes may be used. In fact, almost any reaction where there is a change from gel to sol, or solid to liquid, will find it applicable.

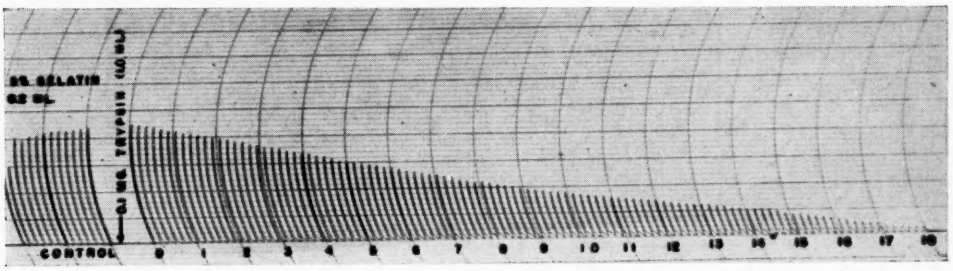

Fig. 3.—Reproduction of a typical permanent record of tryptic activity by use of the apparatus. Each heavy line on ordinate represents ½ Ma., time in minutes on abscissa.

TABLE I. TRYPTIC DECOMPOSITION OF GELATIN, EXPRESSED AS PER CENT DECREASE IN MILLIAMPERES

SUBSTRATE CONC.	SUBSTRATE ML. VOL.	TRYPSIN MG. WT.	TRYPSIN ML. SOLN.	PER CENT DECREASE IN 3 MIN.	PER CENT DECREASE IN 5 MIN.
5 per cent	0.2	0.05	1	9.8	15.9
				0.45*	1.29*
5 per cent	0.2	0.1	1	17.5	29.7
				0.54*	1.07*
5 per cent	0.2	0.2	1	18.4	33.4
				0.70*	1.55*
6 per cent	0.2	0.2	1	15.0	26.4
				0.48*	0.60*

*Standard error.

Table I delineates the results of a series of determinations using 5 per cent and 6 per cent gelatin and 0.05 to 0.2 mg. of trypsin demonstrating typical measurements of proteolytic activity, at 25.1° C. It can be seen that alteration in enzyme concentration or substrate are clearly differentiated. Fig. 5 shows an extrapolation of the curves of the first 3 series over a 15-minute period, revealing that whereas in the lower concentrations the enzyme acts as the limiting factor, in the higher concentrations the substrate becomes the limiting factor. Forty-eight samples were used in the determinations reported, 12 for each condition. Control runs with water or saline replacing the trypsin solution produced negligible alterations in the records over a fifteen to thirty minute period. We have found that the presence of some electrolyte in the substrate aids in the fidelity of the reproduced record.

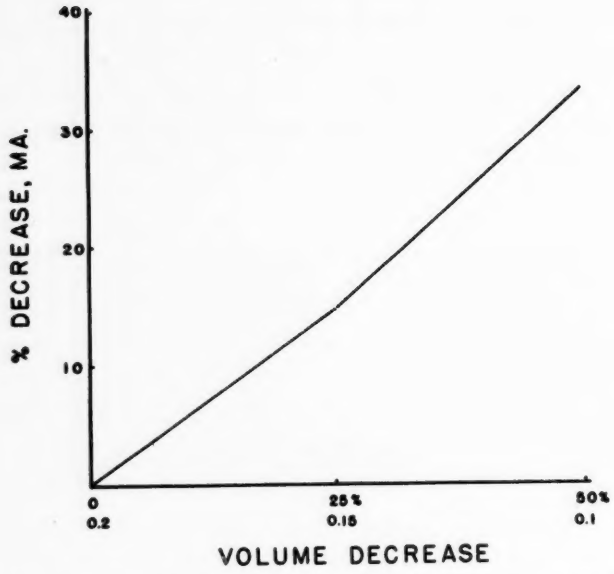

Fig. 4.—Calibration of the alteration of conductivity in milliamperes as a function of volume decrease of substrate.

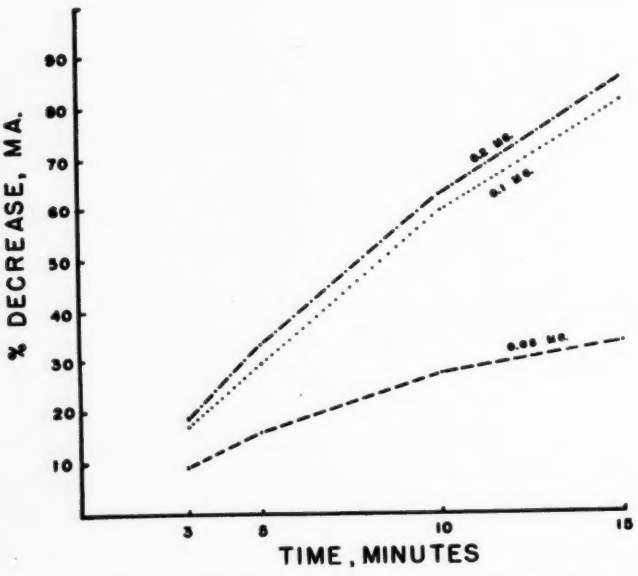

Fig. 5.—The decrease in conductivity of the substrate due to volume decay as a function of time, when 0.05, 0.1, and 0.2 mg. of trypsin were used as the proteolytic enzyme quantities. The original substrate was 0.2 ml. of 5 per cent gelatin.

SUMMARY

It is believed that the new method for proteolytic enzyme activity determinations reported here involves a minimum of subjective error, and offers the advantage of reproducible permanent recordings. These features, along with its simplicity and reliability, should make it a practical and convenient instrument for use in such determinations, as well as with other solid-to-liquid reactions.

We should like to acknowledge our gratitude to Dr. Calvin L. Turbes and Dr. Felix Haurowitz for their technical advice during the enzyme studies.

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A SIMPLIFIED TECHNIQUE FOR BLOOD VOLUME DETERMINATIONS USING I^{131}HSA

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RECENT heavy demands for blood volume determinations on cardiac and other patients at this institution have caused us to simplify and accelerate our determinations. The rationale and uses for this test have been adequately described in the literature.[1-4]

The advent of scintillation counters and disposable plastic containers has considerably decreased the time of performing the tests while reducing radiological hazards. Using similar measuring devices (syringes) for measuring both the known and unknown samples has eliminated the need for accurately calibrated pipettes. Moreover, the increased sensitivity of our detectors has enabled us to use doses of such low concentrations that serial tests can be performed on the same patients at daily or shorter intervals.

METHOD

Material.—

7.5 μc I^{131}HSA*
50 ml. isotonic sodium chloride solution†
2,000 ml. tap water

Apparatus (Fig. 1).—

Scintillation well counter NaI (Th) using modified Morton[5] circuit for 300 volt plateau. Plastic test tubes, ½" O.D., 4" long, with metallic screw cap.‡

1—1 ml. tuberclin syringe (sterile)
1—20 ml. syringe (sterile)
1—5 ml. syringe (heparinized,§ sterile)
1—Wintrobe tube
3—20 gauge needles (sterile)
2—Paper cups (1 ounce)
1—2,000 ml. volumetric flask

Procedure.—

1. Using the tuberculin syringe, 7.5 μc of I^{131}HSA is withdrawn from the commercial bottle under sterile conditions and inserted into the 50 ml. sterile isotonic sodium chloride solution. The volume injected is not critical and is usually less than 0.1 ml.

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*Atomic Energy Commission, Oak Ridge, Tenn., and Abbott Laboratories, North Chicago, Ill.

†Abbott Laboratories, North Chicago, Ill.

‡Lusteroid Corporation, Maplewood, N. J.

§Upjohn Company, Kalamazoo, Mich.

2. Twenty milliliters of the compounded solution are withdrawn by means of a sterile syringe and injected into the antecubital vein.

3. The syringe is rinsed in tap water in the 1 ounce paper cup, and 20 ml. of the prepared solution are withdrawn and made up to a 2,000 ml. volume with tap water.

4. Fifteen minutes after step 2, a volume of 5 ml. of blood is withdrawn with a 5 ml. heparinized syringe and inserted into a disposable plastic test tube.

5. An extra 1 ml. is withdrawn in step 4 to perform a hematocrit test. This is centrifuged at 3,000 r.p.m. for 30 minutes.

6. After rinsing in tap water the syringe and needle used in step 4, a sample of 5 ml. is withdrawn from the 2,000 ml. volumetric flask and similarly injected into another plastic test tube.

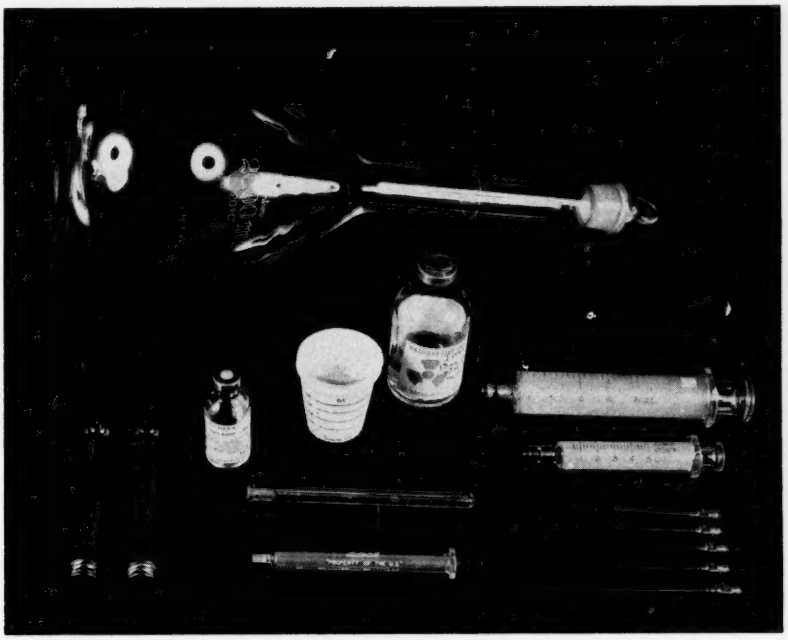

Fig. 1.—Equipment for Blood Volume Determinations.

7. The test tubes prepared in steps 4 and 6 are counted in a scintillation well counter for 5 minutes each. The probable statistical error is less than ±1 per cent for the concentrations employed. A plateau of less than 2 per cent variation per 100 volts over a 300 volt range enables this device to be used on unregulated voltage lines by medical technicians.

8. A mimeographed record sheet is provided and the calculations for total blood volume are made according to the following:

$$\frac{\text{Net counts (std.)}}{\text{Net counts (blood)}} \times 2{,}000 = \text{Blood volume}$$

$$\text{Blood Volume } (1.00 - \text{Hematocrit}) = \text{Plasma volume}$$

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